The regulation of PI 3-kinase and its downstream effectors in T-lymphocyte costimulation

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THE REGULATION OF PI 3-KINASE AND ITS DOWNSTREAM EFFECTORS IN T-LYMPHOCYTE COSTIMULATION.

submitted by

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for the degree of PhD
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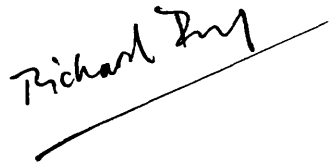

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Abstract.

Ligation of the T cell costimulatory molecule CD28 has previously been demonstrated to induce tyrosine phosphorylation of its cytoplasmic domain and activation of the putative signalling molecule phosphatidylinositol 3-kinase (PI 3-kinase). Here, ligation-induced tyrosine phosphorylation of CD28 is shown to represent only a minor component of phosphorylated residues, whilst heavier phosphorylation resides within serine and threonine residues. The ligation-dependent phosphorylation of CD28 has been characterised here as resistant to known inhibitors of a range of known serine/threonine kinases.

Recruitment of PI 3-kinase to CD28 is dependent upon the phosphorylation of ^{173}Y within the cytoplasmic domain of CD28, but independent of lck, subsequent activation of PI 3-kinase however, is severely impaired in the absence of lck. The CD28-mediated PI 3-kinase signal is also inhibited by the PMA-mediated activation of PKC which disrupts CD28/PI 3-kinase association, and potentially by an SH2 containing inositol polyphosphate 5-phosphatase (SHIP), since CD28 ligation is followed by tyrosine phosphorylation, and stimulation of catalytic activity, of SHIP. *In vitro* protein kinase assays, of SHIP immunoprecipitates, reveal CD28-mediated phosphorylation of SHIP to be accompanied by phosphorylation of unidentified substrates of approximately 40 and 100 kDa, and these may facilitate CD28 regulation of SHIP.

CD28-mediated activation of PI 3-kinase has been demonstrated to stimulate the activity of the downstream effectors p70 S6 kinase and protein kinase B (PKB). Effects of CD28 ligation on other biochemical events such as activation of the MAP kinases JNK and p38 have also been investigated. Finally, ligation of CD95 has been shown to stimulate the activity PI 3-kinase and the downstream effector PKB.

Acknowledgements.

As I alighted into the sharp Northumbrian sunshine, blinking, disoriented -I had but a few thoughts clear in my mind. I'd like to thank Dr Stephen Ward for changing one of those, and for his support, energy, clarity of vision and killer comments, throughout my studies. Thanks must also go to the indefatigable Prof. John Westwick for his enthusiastic input and selfless work in creating a stimulating environment to study in.

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Table of contents. **Page**

Section One: Introduction.

Section Two: Materials and Methods.

Section Three: Early biochemical events regulating recruitment and activation of phosphatidylinositol 3-kinase by CD28.

Section Four: Ligation stimulated phosphorylation of CD28.

Section Five: CD28 activation of downstream effectors and regulators of the PI 3-kinase-dependent signalling cascade.

List of Figures **Page**

Section One

Section Two.

Section Three.

Section Five.

Section Six.

Section Seven.

List of Tables. **Page**

Section One.

Section Two.

Section Four.

Section Six.

Abbreviations.

Ab	Antibody
Ag-MHC	Antigen-major histocompatability complex
AICD	Activation induced cell death
AP-1	Activator prtoein 1
APC	Antigan presenting cell
APS	Ammonium persulphate
ARAM	Antigen recognition and activation motif
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BLV	Bovine leukaemia virus
Ca^{2+}	Calcium ions
CAPS	Cytotoxicity dependent Apo-1 associated proteins
Caspases	Cascade of aspartate specific cysteine proteases
cDNA	Complementary deoxyribonucleic acid
CD	Clusters of differentiation
CDR	Complementary determining region
CD28RE	CD28 response element
ced genes	*Caenorhabditis elegans* death genes
CHO cells	Chinese hamster ovary cells
CPM	Counts per minute
CsA	Cyclosporin A
CTLA-4	Cytotoxic lymphocyte associated antigen 4
DAG	Diacyl glycerol
DED	Death effector domain
DMSO	Dimethyl sulphoxide
DISC	Death inducing signaling complex
DMEM	Dulbecco's minimal essential medium
DNA-PK_{cs}	DNA-dependent protein kinase catalytic subunit
DR	Death receptor
EBV	Eppstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FADD	Fas associated death domain containing protein
FAP1	Fas associated phosphatase 1

FBS	Foetal bovine serum
FITC	Fluoresein isothiocyanate
FLICE	FADD-like ICE
GAP	GTPase-activating protein
Grb-2	Growth factor receptor binding protein
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]
HPLC	High performance liquid chromatography
hr	Human recombinant
ICE	Interleukin -1β converting enzyme
IFNγ	Interferonγ
Ig	Immunoglobulin
IL	Interleukin
IRS-1	Insulin receptor substrate-1
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITK	Inducible T cell kinase
JNK	c-jun N-terminal kinase
kDa	Kilodaltons
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzyopyran-4-one
mAb	monoclonal antibody
MAP kinase	Mitogen activated protein kinase
MIP-1α	Macrophage inflammatory protein-1α
mRNA	messenger ribonucleic acid
NFκB	Nuclear factor-κB
NGFR	Nerve growth factor
NOD	Non obese diabetic
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDK1	PtdIns(3,4,5)P_3-depednent kinase-1
PHA	Phytohaemagglutinin
PI	Propidium iodide
PK A/B/C	Protein kinase A/B/C
PI 3-kinase	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PS	Phosphatidyl serine
PtdIns	Phosphatidylinositol

PTK	Protein tyrosine kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH	*src* homology
SHIP	SH2 containing inositol 5'polyphosphatase
TCR	T cell antigen receptor
TEMED	N,N,N',N'-tetramethylethylendiamine
Th	T helper
TLC	Thin layer chromatography
TNF	Tissue necrosis factor
TOR1/2	Target of rapamycin 1/2
ZAP	Zeta-associated protein
ZVAD-FMK	Cbz-Val-Ala-Asp-(OMe)-fluoromethyl ketone

Single letter amino acid code:

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Lec
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

SECTION ONE

INTRODUCTION

1.1 Background

T lymphocytes play a central role in both the induction and coordination of an acquired immune response, performing diverse functions in recognition of foreign material, cytotoxicity, the facilitation of B cell antibody production and recruitment of accessory cells. Resting T cells circulating in the peripheral blood become activated on encountering processed antigenic peptide in complex with major histocompatibility protein (Ag-MHC) on the surface of an antigen presenting cell (APC). This recognition event is mediated by the T cell antigen receptor and intiates a series of biochemical events enabling the T cell to produce a range of cytokines including IL-2, and express the IL-2 receptor. It is now generally accepted however, that the biochemical signals elicited by the T cell antigen receptor alone, are insufficient to allow for optimal IL-2 production, and that the presence of a second, or costimulatory, signal is required [Bretscher and Cohn (1970)]. This second signal is provided by molecules present on "professional" antigen presenting cells, such as dendritic cells, monocytes and activated B cells.

The interaction of CD28 with its natural ligand B7, has emerged as the major source of costimulation in T cells. In combination, the signals transduced by the T cell antigen receptor and CD28 stimulate optimal IL-2 production, allowing for paracrine and autocrine driven progression through the cell division cycle (proliferation). Lymphocytes that are activated by ligation of the T cell receptor alone may enter a state of antigen unresponsiveness, termed anergy, or undergo death by apoptosis. Apoptosis, or programmed cell death, is a form of cell suicide which is believed to play an important role in development, tissue homeostasis and clearance of damaged cells from multicellular organisms [Steller (1995)]. The presence of the CD28-mediated costimulatory signal, effectively drives the cell away from apoptosis, highlighting a role for CD28 in cell survival, towards a proliferative response [Mueller *et al.* (1989)]. CD28 can thus be regarded as a pivotal signal in T cell activation. The signals elicited by CD28 must be at least partially distinct from those transduced by the T cell antigen receptor, since the former signal is classically described as resistant to cyclosporin A and FK506 [June *et al.* (1987)]. Given these functional and biochemical distinctions it is important to understand the differences between the signals transduced the TCR and CD28.

1.2.1 Structure of the T cell antigen receptor

In any study of costimulatory signalling, it is important to consider the cellular environment within which these signals are elicited. Effectual T cell activation is believed to begin with the recognition of MHC complexed antigenic peptide expressed on the surface of an antigen presenting cell (APC). Recognition of the Ag-MHC complex is mediated by the T cell antigen receptor (TCR). The TCR is a complex oligomer composed of the products of six genes, all of which are required for efficient plasma membrane expression. A ligand binding site, formed by an α and β chain (or less frequently a γ and δ chain) is responsible for the recognition of Ag-MHC. The transmembrane and membrane proximal moieties are responsible for interchain associations and assembly of the oligomeric structure, whilst CD3 and TCR ζ chains are responsible for signal transduction.

hTCRζ1	QL	Y	NE	L	NLGRREE-	Y	D	V	L
hTCRζ2	GL	Y	NE	L	QKDKMAEA	Y	S	E	I
hTCRζ3	GL	Y	Q	L	STATKDT-	Y	D	A	L
hCD3γ	QL	Y	QP	L	KDREDDQ-	Y	SH		L
hCD3δ	QV	Y	QP	L	RDRDDAQ-	Y	SH		L
mCD3ε	PD	Y	EP	I	RKGQRDL-	Y	SG		L
mIgα	NL	Y	EG	L	NLDDCSM-	Y	ED		I
hIgE FcRγ	GV	Y	TG	L	STRNQET-	Y	ET		L
BLV gp30	SD	Y	QA	L	LPSAPEI-	Y	SH		L
EBV-LMP2A	I	Y	SH	I	SPVKPD--	Y	IN		L

Figure 1.1 Structure of the immunoreceptor tyrosine-based activation motif (ITAM). ITAMs are expressed in T and B cell antigen receptors, the mast cell receptor for IgE, and in the viral glycoproteins of bovine leukaemia virus (BLV) and Epstein-Barr virus (EBV) latent membrane protein 2A.

The principal structural unit which regulates TCR coupling to downstream signal transduction is the immunoreceptor tyrosine-based activation motif (ITAM) or alternatively the antigen recognition and activation motif (ARAM), first described by Reth (1989). These are present as single copies in each of the CD3 chains and triplicated in the

ζ chain. Each ITAM consists of two YXXL/I repeats separated by 6-8 residues, this arrangement is represented in Figure 1.1 from sequences in proteins expressing ITAMs. The tyrosine residues of the ITAM undergo ligation dependent phosphorylation. The multiplicity of the ITAMs within the TCR is striking, since each TCR can contain a total of 10 ITAMs. One function of this multimerisation of the motif is thought to be signal amplification. This is an attractive hypothesis since relatively few Ag-MHC molecular complexes are available on APCs. Thus, a single recognition event could involve the participation of multiple ITAMs that each interact with intracellular signalling molecules.

1.2.2 Biochemical events elicited by the T cell antigen receptor.

Signal transduction events following ligation of the TCR (termed signal 1) are mediated by the cytoplasmic domains of the CD3 complex, by the co-receptor CD4 or CD8 and by accessory molecules such as CD45. An early event associated with signal 1 is the phosphorylation of several protein substrates. Three distinct protein tyrosine kinases (PTKs) have been implicated in TCR function: p56*lck*, p59*fyn* and ZAP-70. Lck is a T cell restricted member of the *src* family PTKs and is associated at a high stochiometry with the co-receptors CD4 and CD8. The co-receptors may function to increase the overall avidity of the interaction of the TCR with Ag-MHC. Alternatively it is postulated that simultaneous binding of the TCR and CD4 or CD8 co-receptor results in the juxtaposition of p56*lck* and CD3 cytoplasmic domains, facilitating their phosphorylation. Expression of a constitutively activated (^{505}Tyr to ^{505}Phe mutant) form of lck in a CD4 negative mouse T cell hybridoma increases TCR stimulated tyrosine phosphorylation of proteins and its sensitivity to antigenic stimulation [Abraham *et al.* (1991)]. A critical function for lck in TCR signal transduction is also suggested by genetic studies. A mutant leukaemic cell line selected for its inability to increase cytoplasmic free calcium (a consequence of phospholipase C γ1 activation) in response to anti-CD3 mAb, is unable to activate the TCR PTK pathway. The defect in this mutant was shown to represent its failure to express functional lck [Straus and Weiss (1992)].

Another member of the *src* family PTKs, namely p59^{fyn}, was implicated in TCR signalling when fyn kinase activity was detected in TCR immunoprecipitates using *in vitro* kinase assays [Samelson *et al.* (1990)]. The authours did acknowledge however, that this may have been an indirect association. Additionally TCR stimulation is followed by rapid but transient 2-4 fold increase in *fyn* kinase activity [Tsygankov *et al.* (1992)]. The N-terminal 10 amino acids of *fyn* have been shown to associate with distinct regions

of the ζ chains [Gauen *et al.* (1992)], however the stoichiometry of *fyn* association appears to be quite low.

The ability of CD8/TCR ζ chimaeras, containing the ζ cytoplasmic domain, to induce cellular tyrosine phosphorylation, suggested that ζ might interact directly with a PTK [Chan *et al.* (1991)]. Immunoprecipitates of ζ from stimulated cells have been shown to contain a tightly associated 70 kDa protein named ZAP-70 [Chan *et al.* (1991)]. Further characterisation of ZAP-70 has revealed its expression to be restricted to T cells and NK cells. It has two N-terminal SH2 domains and a C-terminal kinase domain. ZAP-70 is most homologous to Syk, a 72 kDa PTK abundant in B cells and myeloid cells, which may associate with the B cell antigen receptor. The association of ZAP-70 with phosphorylated CD3 ζ is rapid and specific.

Several cellular substrates have been identified for TCR-activated PTKs, including components of at least two signalling pathways believed to be important links in the control of cell activation. The first of these is phospholipase Cγ1 (PLCγ1) [Park *et al.* (1991)], which regulates the hydrolysis of membrane phospholipids, namely PtdIns(4,5)P_2, yielding two important products, Ins(1,4,5)P_3 and diacylglycerol (DAG). Ins(1,4,5)P_3 regulates intracellular calcium release [Berridge (1993)], whilst DAG activates the serine/threonine kinase protein kinase C (PKC) [Nishizuka (1998)]. Secondly, TCR activation of the guanine nucleotide binding protein $p21^{ras}$ is PTK-dependent and is essential for the coupling of the TCR/CD3 complex to mitogenic-activated protein kinase (MAP kinase), also termed extracellular-signal-regulated kinase 1/2 (ERK1/2) [Izquierdo *et al.* (1995)]. The calcium signalling system cooperates, via the calcium phosphatase calcineurin, with $p21^{ras}$ for induction of the transcription factor nuclear factor of activated T cells (NFAT) which, along with nuclear transcription factor κB (NFκB), AP-1 and Oct-1, is involved in IL-2 gene expression [Izquierdo *et al.* (1995)]. A third signalling pathway, which is putatively mediated by PI 3-kinase, has also been implicated in cell growth and activation. This may also be controlled by TCR-activated PTKs, since the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) binds to the TCR ζ chain, although this has not been consistently observed to correlate with PI 3-kinase activation [Ward *et al.* (1992); Carrera *et al.* (1994)]. The biochemical signals comprising signal 1 are summarised schematically in the Figure 1.2.

Following the identification of the necessity of a costimulatory signal, several possibilities were proposed as to the biochemical nature of this signal: i) it could be a unique signal activating distinct signal transduction pathways which complement those activated by the TCR, ii) it could correspond to signal 1 but occur at different time points or in different cellular compartments, or iii) it could be the same as signal 1 but act in an additive fashion, enhancing the amplitude or duration of signal 1, allowing activation thresholds to be crossed and activation of downstream targets. Subsequent investigations have revealed elements of signal 2 to correspond with each of these hypotheses, for example both signals 1 and 2 result in the phosphorylation of the adaptor proteins p95vav [Tuosto *et al.* (1996); Klasen *et al.* (1998)] and SLP76, whilst only signal two has been reported to induce phosphorylation of p62dok [Klasen *et al.* (1998)]. Some of these features of TCR-mediated and co-stimulatory signalling are summarised below and discussed in section 1.5.

Signalling event	Signal 1 (TCR/CD3)	Signal 2 (CD28)
Activation of PTK's	p56lck, p59fyn [Tsygankov *et al.* (1992)], ZAP-70 [Chan *et al.* (1991)]	p56lck, p59fyn, ITK [August and DuPont (1994)]
PTK substrates	PLCγ1 [Straus and Weiss (1992)]	PLCγ1 activation in response to anti-CD28 Ab [Nunes *et al.* (1993)] but not B7.1 [Ward *et al.* (1993)].
Phosphorylation of adaptor proteins	p95vav [Tuosto *et al.* (1996)]. SLP76 p36 LAT [Zhang *et al.* (1998)]	p95vav [Klasen *et al.* (1998)]. SLP76
Ceramide	Not reported	A-SMase [Boucher *et al.* (1995)].
SHIP activation	Via TCRζ [Daeron *et al.* (1995)].	Tyrosine phosphorylation and activation (this study).
MAP kinase activation	Activation of JNK reported as	synergistic in response TCR and CD28 ligation [Su *et al.* (1994)]

Table 1.1 Signalling events elicited following TCR and CD28 ligation.

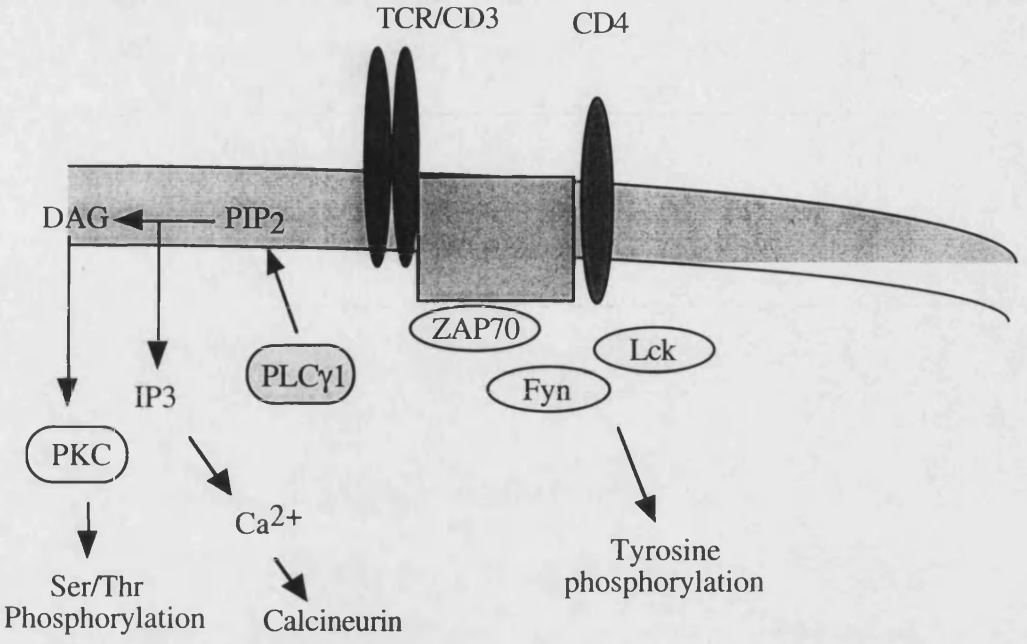


Figure 1.2 Schematic summary of signalling events comprising signal 1. TCR ligation induces the activation of src-family PTKs lck and fyn. Substrates include PLCγ1. When phosphorylated PLCγ1 catalyses the breakdown of PtdIns(4,5)P_2 forming DAG which activates PKC and Ins(1,4,5)P_3 which regulates intracellular calcium levels.

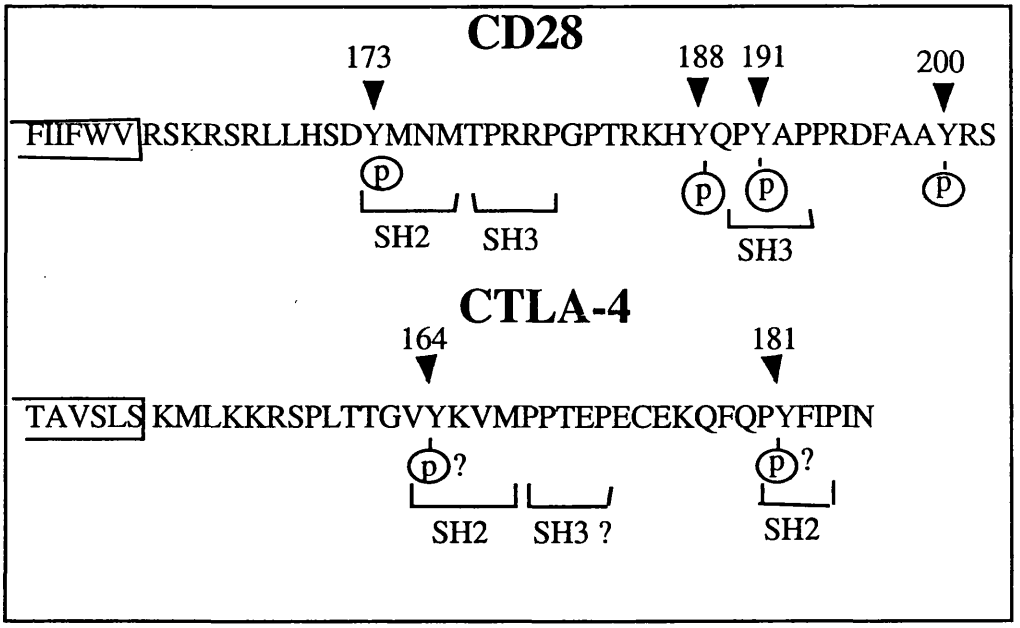
1.3 Two signal model: The concept of costimulation.

It is now accepted that signalling events intiated by the TCR alone are insufficient to allow for optimal IL-2 production and proliferation. For a T cell to support sufficient IL-2 production to allow for clonal expansion, there is a further requirement for costimulatory signals, additional to those produced on TCR/Ag-MHC ligation. This observation was summarised nearly thirty years ago by Bretscher and Cohn (1970), in their two signal model of signal transduction. In its simplest form the model, as originally proposed, describes that an individual APC can independently express the Ag-MHC complex (transducing signal 1), and/or a costimulatory ligand (transducing signal 2). Thus, given the binary nature of the model it is possible to predict three potential outcomes of the interactions of APCs and T cells: i) When resting cells encounter costimulatory ligand in the absence of Ag-MHC, no effects are observed; ii) the interactions of T cells with Ag-MHC in the absence of costimulatory ligand is not a neutral event but leads to a state of induced Ag unresponsiveness termed anergy, or to cell death by apoptosis; iii) activation of the TCR in the presence of costimulatory signals results in T cell clonal expansion and induction of effector functions. Thus, in the third case the presence of the costimulatory signal is driving the T cell away from apoptosis or anergy, towards effector function.

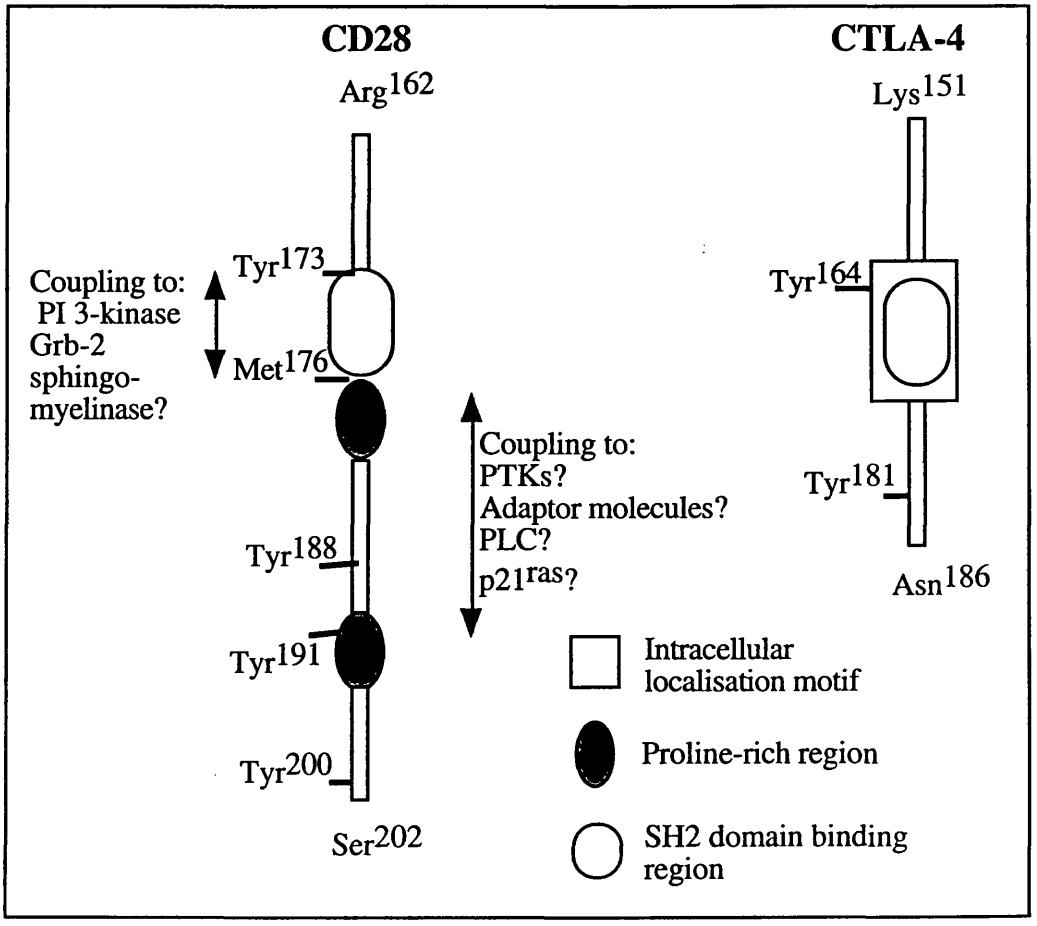
1.4 The CD28/CTLA-4 "family".

The first evidence that CD28 could intiate costimulatory signals in resting human T cells came from the observation that, in conjunction with phorbol-12-myristate-13-acetate (PMA), CD28 induced cyclosporin A-resistant T cell proliferation and IL-2 production [Hara *et al.* (1985); June *et al.* (1987)]. Further studies led to the identification of an additional "family" member namely, CTLA-4. The genes for CD28 and CTLA-4 are closely linked on human chromosome 2 (2q33-34) and display a similar genomic organisation, implying a common evolutionary origin [Harper *et al.* (1991)]. Despite this genomic relationship, the overall amino acid conservation between the two molecules is only 30%. CD28 is a homodimeric glycoprotein expressed on 95% of $CD4^+$ T cells and approximately 50% of $CD8^+$ T cells [Damle *et al.* (1983)]. Although the extracellular domains display little sequence identity, mapping of the ligand binding sites of CD28 and CTLA-4 has localised the site of interaction with B7.1 and B7.2 to a conserved sequence M-Y-P-P-P-Y in the complementary determining region 3-like region, (CDR3)-like region, of both CD28 and CTLA-4. The amino acid sequences of the CD28 and CTLA-4 cytoplasmic tails are given in Figure 1.3. CD28 is composed of two glycosylated 44 kDa chains, which are members of the immunoglobulin super-family each containing a single disulphide linked extracellular Ig V-like domain. The CD28 polypeptide contains 202 amino acids giving a molecular mass of 23 kDa which is then glycosylated to the molecular mass of the mature protein. The extracellular domain is linked, via a single pass transmembrane region, to a 41 amino acid cytoplasmic domain which is responsible for initiating costimulatory signals. The cytoplasmic domain of CD28 does not encode any recognised motif for kinase activity, and is therefore presumed to signal via the recruitment of cellular enzymes. Notably, there is a consensus sequence motif, ^{173}(p)YMNM, within the cytoplasmic tail of CD28 which binds SH2 domains of the p85 subunit of phosphatidylinositol 3-kinase [Pages *et al.* (1994)] and growth-factor-receptor binding protein (Grb-2), a ubiquitous adaptor protein [Schneider *et al.* (1995a)]. Additionally, CD28 also contains two proline rich motifs which conform to the P-X-X-P SH3 consensus binding sequence [Ren *et al.* (1992)], and this may allow for CD28 interaction with further signalling proteins.

CTLA-4 displays a similar structure to CD28 as a disulphide linked homodimer of predicted molecular mass of 20 kDa with a single glycosylation site. This protein also consists of a single disulphide linked extracellular IgV like domain linked via a short amino acid stretch to a transmembrane region and cytoplasmic domain of 36 amino acids. There is only limited sequence homology (approximately 30% at the amino acid level) between the cytoplasmic domains of CD28 and CTLA-4, possibly suggesting an ability



A)



B)

Figure 1.3 Schematic summary of CD28 and CTLA-4 cytoplasmic domains. [Amended from Ward (1996)]. **A)** Amino acid sequence of CD28 [Aruffo and Seed (1987)] and CTLA-4 [Harper *et al.* (1991)] is given, with potential interactions with signalling domains indicated. **B)** Schematic summary of functional domains of CD28 and CTLA-4 cytoplasmic tails.

to generate different signals. Although the biochemical signals generated by CTLA-4 are poorly defined, CTLA-4 does contain a consensus site for PI 3-kinase binding and anti-CTLA-4 mAbs have been reported to induce PI 3-kinase binding to this site [Schneider *et al.* (1995a)]. CTLA-4 is not constitutively expressed but expression is induced following T cell activation by TCR and CD28 ligation [Alegre *et al.* (1996)]. The functional role of CTLA-4 is currently the subject of intense investigation which, to date, has yielded conflicting reports. Both amplification [Jenkins (1994)], and suppression [Walunas *et al.* (1994)] of the T cell immune response has been postulated. Studies of gene-targeted mice lacking CTLA-4 however, demonstrate a progressive accumulation of T cell blasts, indicating CTLA-4 as a negative regulator [Waterhouse *et al.* (1995)]. The intra-cytoplasmic domains of CD28 and CTLA-4 are represented schematically in Figure 1.3.

1.4.1 Functional outcomes of CD28 ligation.

Ligation of CD28 alone has little effect on resting T cell proliferation, however CD28 ligation in the presence of limited concentrations of anti-CD3 Ab promotes T cell proliferation and IL-2 production by regulating IL-2 mRNA at the level of both transcription and mRNA stabilisation. Costimulation via CD28 also mediates strong upregulation of the IL-4, IL-5, IL-13, γ-interferon, tumour necrosis factor α and granulocyte/ macrophage colony-stimulating factor (GM-CSF) [Thompson *et al.* (1989); Minty *et al.* (1993); Seder *et al.* (1994)], up regulation of the IL-2 receptor α and β chains, and upregulation of CD40 ligand expression. Up-regulation of the chemokines IL-8 [Wechsler *et al.* (1994)], RANTES [Turner *et al.* (1995)] and MIP-1α [Herold *et al.* (1997)] have also been reported following CD28 stimulation. Two transcription factors namely, activator protein (AP)-1 and nuclear factor-κB (NF-κB), have been suggested to be influenced by CD28, enabling such transcriptional effects [Edmead *et al.* (1996)]. The mechanisms by which CD28 regulates AP-1 and NF-κB have not yet been defined however, studies with inhibitors suggest that they may involve activation of PI 3-kinase and generation of ceramide repectively [Edmead *et al.* (1996)].

CD28 not only enhances IL-2 production, which can act as an extrinsic survival factor, but also augments the expression of the intrinsic cell survival factor Bcl-X_L [Boise *et al.* (1995)]. Furthermore, protein kinase B appears to be regulated by the lipid products of PI 3-kinase [Franke *et al.* (1997); Stokoe *et al.* (1997)], and thus may be influenced by CD28. PKB provides a signal that is protective of cells from apoptosis induced by various stresses, which may correlate with its amplification in many human tumours [Cheng *et al.* (1996)].

1.4.2 The CD28 response element

The IL-2 gene is the best characterised of those cytokine genes affected by CD28. The 5' upstream region of the IL-2 promoter, between -325 and the transcriptional start site, contains a number of binding sites for transcriptional factors, including AP1, NFκB, NFAT and OCT [Fraser *et al.* (1993)], for transcriptional activation. The binding of these proteins to their recognition sites within the IL-2 promoter upregulates IL-2 gene transcription. The CD28 response element (CD28RE) is located within the IL-2 promoter between -160 and -152 relative to the transcription start site. Mutation of the CD28RE, which appears to be related to, but distinct from the NFκB element, results in the loss of CD28 induced activity without affecting the TCR induced IL-2 promoter activities [Fraser *et al.* (1992a)]. In common with IL-2, several other lymphokines have conserved sequences within their promoters and studies have indicated that the GM-CSF, IL-3 and IFN-γ promoters are responsive to CD28 signal transduction [Fraser *et al.* (1992b)].

1.4.3 The B7 family.

Ligands for CD28 and CTLA-4 have been identified as B7.1 [Freeman *et al.* (1989)] and B7.2 [Azuma *et al.* (1993)]. B70 is an alternate splice variant of B7.2 that is identical to B7.2, except for six additional amino acids at the N terminus of the B7.2 sequence [Azuma *et al.* (1993)]. Additionally, there is evidence for a further B7 molecule provisionally termed B7.3 [Boussiotis *et al.* (1993)], although this molecule has never been cloned or characterised. B7.1 and B7.2 are members of the immunoglobulin superfamily with two Ig like domains (Ig-C and Ig-V), although they share only 25% sequence homology. B7.1 is a 60 kDa glycoprotein consisting of the two extracellular disulphide-linked Ig-like domains, a transmembrane region and a 19 amino acid cytoplasmic domain. B7.2 is a 70 kDa glycoprotein whose major feature is an extended cytoplasmic region which contains potential PKC phosphorylation sites.

1.4.4 Evidence that B7.1 and B7.2 differentially regulate the immune response.

A number of differences have been reported between B7.1 and B7.2 which suggest these molecules differentially regulate the immune system: i) Whilst both B7.1 and B7.2 are

expressed on activated monocytes, activated B and T cells and activated natural killer cells, only B7.2 is expressed constitutively on dendritic cells and resting monocytes [Lenschow *et al.* (1993)]. ii) B7.2 expression on human B cells activated by immunoglobulin crosslinking peaks within 24 hours, whilst maximal expression of B7.1 is observed more than three days later [Boussiotis *et al.* (1993)]. iii) In contrast to anti-B7.1 mAbs, anti-B7.2 mAbs are potent inhibitors of T cell proliferation and cytokine production *in vitro* [Lenschow *et al.* (1993); Chen *et al.* (1994)]. iv) B7.1 is superior to B7.2 costimulation in the induction and maintenance of T cell-mediated anti-leukaemia immunity [Matulonis *et al.* (1996)]. v) The dissociation of the B7.2/CTLA-4 complex occurs much faster than the B7.1/CTLA-4 complex [Linsley *et al.* (1994)]. Thus the fast on-off nature of B7.2/CTLA-4 interactions may function to, limit an immune response from the high avidity CTLA-4 receptor. In support of the hypothesis that B7.1 and B7.2 can regulate different functions, *in vivo* studies have shown that the molecules may play distinct roles in the differentiation of $CD4^+$ Th cells. Th0 cells can differentiate into either Th1 cells (which produce IL-2, γ-interferon and TNF-α, and mediate cell-mediated immune responses) or Th2 cells (which produce IL-4, IL-5 and IL-10 and which regulate humoral immune responses). It has been proposed that Th2 immune responses are dependent on high doses of initial antigen and CD28 costimulation, whereas Th1 immune responses are less dependent on CD28 costimulation. Hence reports have shown that B7.1 preferentially acts as a costimulator of Th1 cells, where as B7.2 costimulates and induces Th2 cells [Kuchroo *et al.* (1995)]. The molecular basis for the distinct regulation of differentiation is obscure since there are no significant differences reported in the ability of B7.1 and B7.2 to activate second messengers.

1.4.5 Therapeutic implications of CD28

Since effective T cell activation can orchestrate the induction of a coordinated immune response, there is great therapeutic potential for intervention at this point. Accordingly a number of trials have demonstrated a potent immunosuppressive effect associated with blocking T cell costimulation. This approach has been successfully applied to transplant biology in the prevention of graft versus host disease (GVHD) [Turka *et al.* (1992); reviewed by Guinan *et al.* (1994)], arthritis [Webb *et al.* (1996)], asthma [Tsuyuki *et al.* (1997)], and multiple sclerosis [Winhagen *et al.* (1985)]. Conversely, blockade of negative regulatory pathways by antibody blocking of the CTLA-4 receptor, can also be used to enhance immune responses for example in tumour therapy [Leach *et al.* (1996)]. Additional to these reports, some dramatic studies have revealed an antiviral effect as a result of CD28 costimulation [Levine *et al.* (1996)]. Furthermore, CD28 costimulation of

$CD4^+$ T cells may confer some resistance to T cell infection by human immunodeficiency virus (HIV) type 1 [Riley *et al.* (1997)].

Despite the successes in GVHD prevention and anti-tumour therapy, unpredicted outcomes have arisen during trials, such as the induction of both disease suppression and exacerbation following the administration of anti CD80/CD86 antibodies to non-obese diabetic (NOD) mice [Lenschow *et al.* (1995); Lenschow *et al.* (1996)]. These findings reinforce the need for fundamental research in this area since the current hypotheses do not adequately explain all the data. A better understanding of the events which lead to T cell activation is therefore essential both for the identification of defects which lead to various diseases, and for the successful design of therapies to treat these conditions.

1.5.1 Phosphatidylinositol 3-kinase dependent signalling cascades: Background and discovery of phosphatidylinositol 3-kinase.

PI 3-kinase was first isolated from cells, associated with the virally encoded PTK, *v-src* [Sugimoto *et al.* (1984)]. Since this lipid kinase activity copurified, and was inhibited in parallel, with tyrosine kinase activity, the two properties were thought to be intrinsic activities of the same polypeptide [Sugimoto *et al.* (1984)]. The relationship between PI 3-kinase and PTKs was further clarified by the observation that the PDGF receptor, which possesses intrinsic PTK activity, could recruit PtdIns 3-kinase within less than a minute after PDGF stimulation [Whitman *et al.* (1987)]. Interest in the PI 3-kinase preceeded the discovery that it functioned in a new pathway, since association of this enzyme with the polyoma virus middle T antigen tightly correlated with cell transformation. Mutants of middle T antigen that failed to associate with the PI 3-kinase, also failed to transform 3T3 fibroblasts [Courtneidge and Heber (1987)].

The first full characterisation of PI 3-kinase came from the purification and cDNA cloning of the enzyme from bovine brain [Otsu *et al.* (1991); Hiles *et al.* (1992)]. This revealed the enzyme to consist of two subunits: a regulatory protein termed p85α; and a catalytic subunit p110α. The structure of the heterodimer is summarised in Figure 1.4. The p85α subunit contains two SH2 domains that interact with a phosphotyrosine based binding motif pYXXM [Pawson *et al.* (1995)], allowing p85α to interact with activated receptors or other tyrosine-phosphorylated molecules which contain this motif. The p85α subunit also contains an SH3 domain which may mediate protein-protein interactions through specific recognition of proline rich sequences [Ren *et al.*(1995)]. Two proline rich regions have also been defined on p85α which border the breakpoint cluster region

(BCR). Interestingly, activation of PI 3-kinase via the SH3 domains binding to one proline-rich region (residues 84 to 99) of p85 has been reported [Pleiman *et al.* (1994)], although it is not clear how this mechanism operates. The BCR has limited homology to the GAP (GTPase activating protein) domain of the BCR gene product. Although BCR domains have been identified in a number of proteins which display GAP activity towards the Rho family GTPases, no equivalent biochemical activity has been found for the BCR domain of p85 [Fry *et al.* (1992)]. The inter SH2 region of p85α is predicted to adopt a helical conformation, possibly positioning the two SH2 domains for optimal interaction with target proteins. This region also contains the p110α binding site. The p110α subunit has a corresponding interaction site for p85α at the amino terminus (residues 20-108). The catalytic domain of p110α is situated at the carboxy terminus. Several features of this domain are conserved in protein kinases, such as the D-F-G motif which forms part of the ATP binding motif. The phosphoinositide kinase (PIK) domain, which lies outside the catalytic domain, is conserved amongst all PI 3- and PI 4-kinases [Flanagan *et al.* (1993)], and although its role is presently unclear, it may be involved substrate presentation.

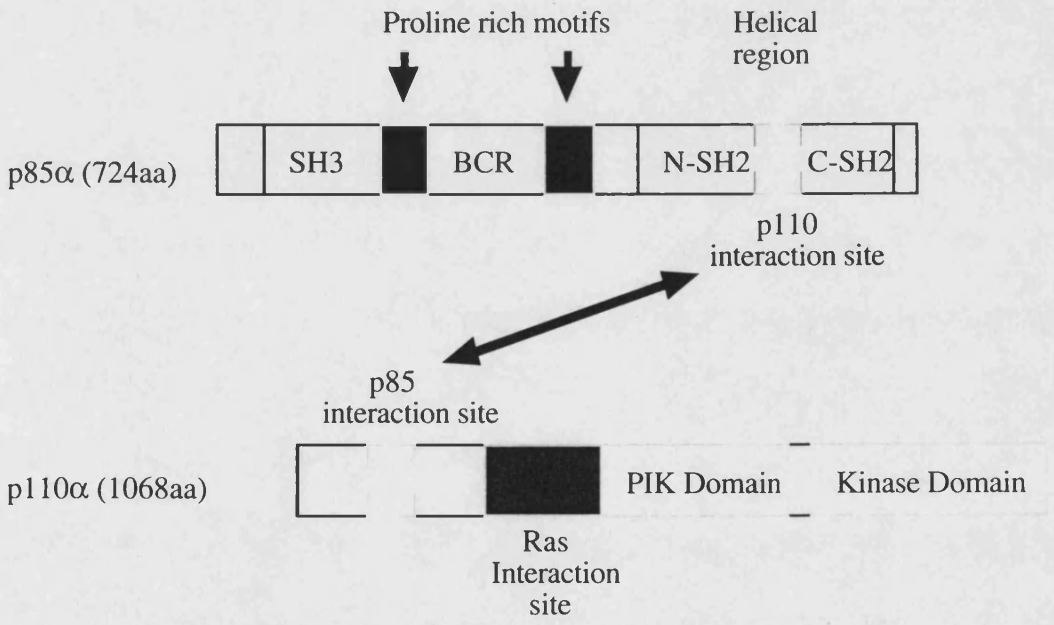
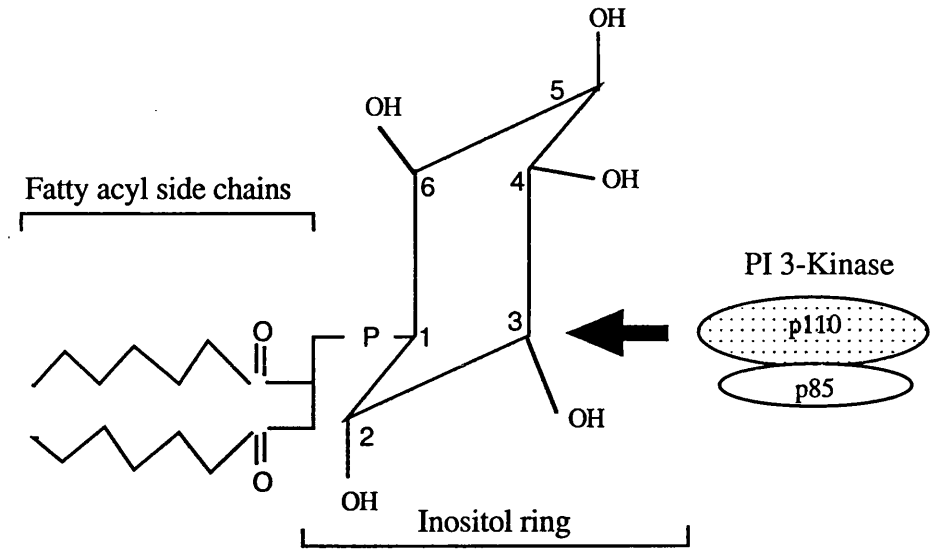
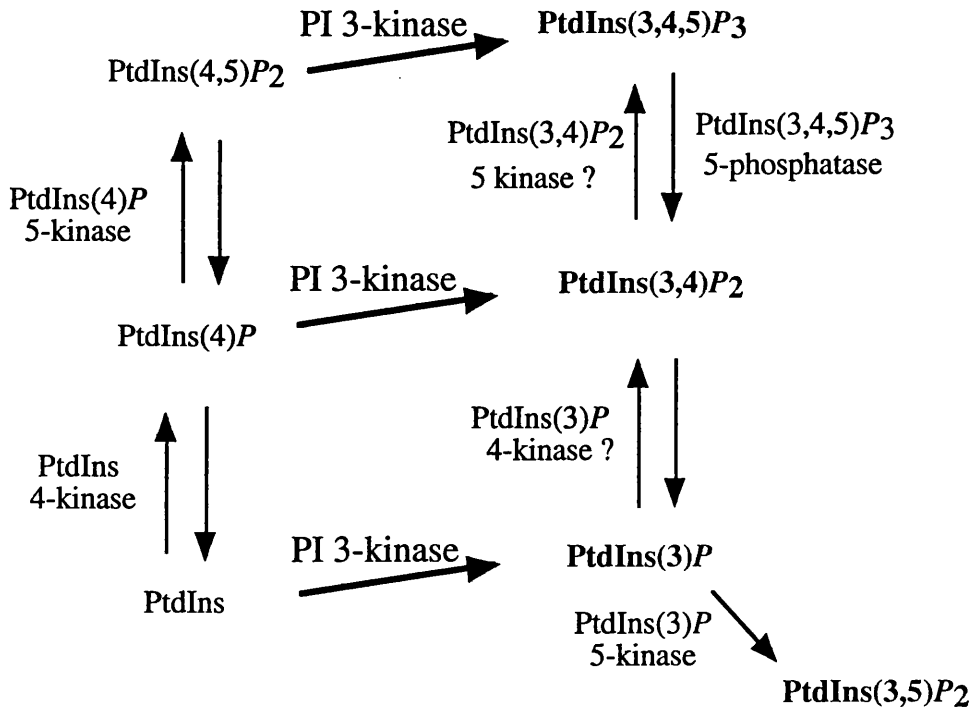


Figure 1.4 Schematic representation of PI 3-kinase.

The lipid kinase activity of the enzyme results in the transfer of a terminal phosphate group from ATP to the D-3 position of the inositol ring of phophatidylinositol lipids. Thus PI 3-kinase can potentially generate three products: PtdIns(3)*P*, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, collectively termed the D-3 phosphoinositide lipids. Considerable importance has been attached to the generation of these compounds, given their putative role as regulatory molecules. Kinetic and stoichiometric evidence indicates that



a)



b)

Figure 1.5 The synthesis of D-3 phosphorylated lipids. a) The structure of PtdIns is represented, with the numbering of the inositol ring and point of phosphorylation by PtdIns 3-kinase indicated. b) Pathways leading to formation of D- 3-phosphorylated inositides are represented. D-series enantiomers are so called since their phosphodiester phosphates are analogous to that in D-Ins1*P*. PtdIns(3)*P* may facilitate roles in sorting of vesicular proteins and PtdIns(3,5)P_2 may play a role in an osmoregulatory pathway [Dove *et al.* (1997)]. Further enzymes contributing to the formation and breakdown of these compounds are also shown.

PtdIns(3,4,5)P_3, formed by a PtdIns(4,5)P_2-specific 3-kinase, is the probable major mediator of intracellular events [Stephens *et al.* (1991)]. The molecular structure of PtdIns, and pathway of phosphoinositide synthesis are represented in Figure 1.5. Phospholipid products of PI 3-kinase recieved attention when a highly polar phospholipid was identified in human neutrophils, levels of which increased rapidly in response to fMLP stimulation [Stephens *et al.* (1991)]. From the chromatographic properties of the head group, this molecule was identified as PtdIns(3,4,5)P_3 [Traynor-Kaplan *et al.* (1988)]. The identification of a second 3-phosphorylated lipid, namely PtdIns(3,4)P_2 in the same cells [Traynor-Kaplan *et al.* (1989)], suggested a connection between PI 3-kinase and these lipids, which was further defined by the demonstration that PDGF not only stimulated the translocation of PI 3-kinase to its receptor but could also induce rapid accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 which were absent from unstimulated cells. Since its discovery, phosphatidylinositol 3-kinase (PI 3-kinase) has been widely implicated in signalling cascades and cellular functions, some of which are summarised in Table 1.4.

In contrast to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, cellular levels of PtdIns(3)P remain relatively constant on receptor stimulation [Auger *et al.* (1989)], indicating that the molecule may not play a role in signal transduction. The yeast protein Vps34, which displays sequence homology with the kinase domain of p110, only phosphorylates PtdIns to PtdIns(3)P [Volinia *et al.* (1995)], and is known to be involved in vacuolar protein sorting. This has led to the belief that PtdIns(3)P is involved in vesicular trafficking of proteins.

The p110 subunit of PI 3-kinase displays a unique dual specificity as both a lipid and protein serine kinase [Dhand *et al.* (1994)]. There is limited knowledge of the significance of the protein serine kinase activity of the enzyme although, substrates are known to include ^{608}Ser on the p85α subunit [Carpenter *et al.* (1993)] which inhibits lipid kinase activity, and IRS 1 [Lam *et al.* (1994)], implying a role in insulin signalling.

1.5.2 The PI 3-kinase family.

Since the intial characterisation of PI 3-kinase [Otsu *et al.* (1991); Hiles *et al.* (1992)], a family of related proteins has emerged. At least three biochemically distinct PI 3-kinases have been described in mammalian cells [Reviewed by Ward *et al.* (1996) and Vanhaesebroeck *et al.* (1997)]: 1) Class I which have a broad subtsrate specificity for

Class	Catalytic Subunit	Schematic Representation	Substrate Specificity	Adaptor/ Binding partner
IA	p110α			p85α
	p110β		PtdIns,	p85
	p110δ		PtdIns(4)*P*, PtdIns(4,5)P_2	p55γ/p55^{PIK}
IB	p110γ			p101
II	PI 3K-C2α/ mcpk/p170		PtdIns, PtdIns(4)*P*,	
	PI3K-C2β			Unknown
	PI 3K_68D /cpk		(PtdIns(4,5)P_2)	
III	Vps34p /PtdIns 3-kinase		PtdIns	Vps15p/ p150

 Kinase domain  PIK domain  C2 domain

 Proline rich domain  ras binding  p85 binding.

Table 1.2: The classification of PI 3-kinase family members. [Amended from Domin and Waterfield (1997)]. Members of the PI 3-kinase family display heterogeneity with respect to subunit compostion and substrate specificity, these are summarised above. The assignment of catalytic subunits to a particular class is based on sequence homology within the catalytic domain [Zvelebil *et al.* (1996)]. To date, PI 3-K-C2α is the only Class II member which has been shown to phosphorylate PtdIns(4,5)P_2. PI3K_68D is a *Drosophila* protein whilst m-cpk and p170 are murine proteins. The yeast enzyme Vps34p and its human homologue PtdIns 3-kinase can only phosphorylate PtdIns [Volinia *et al.* (1995)]. Ths C2 domain was originally defined as the second of four regions within mamalian PKC where it conferred a Ca^{2+} sensitive phospholipid binding [Kaibuchi *et al.* (1980)].

PtdIns and PtdIns(4)*P* and PtdIns(4,5)P_2. This class of enzymes can be further subdivided into Class IA and Class IB. Class IA PI 3-kinases consist of the p110 catalytic subunits that are regulated by the SH2/SH3-domain containing p85 family of adaptor subunits just described. Class IB PI 3-kinases consist of p110γ, an enzyme that associates with a p101 adaptor protein and is stimulated by G protein βγ subunits. 2)

Class II PI 3-kinases are larger enzymes (>200 kDa) that phosphorylate PtdIns and PtdIns(4)*P* but not PtdIns(4,5)P_2. 3) Class III PI 3-kinases which phosphorylate only PtdIns. Unless otherwise stated, the term PI 3-kinase will be used henceforth, to identify the p85/p110 heterodimeric PI 3-kinase. The classes of PI 3-kinases are summarised in Table 1.2.

There is considerable heterogeneity within the family of PI 3-kinases with respect to their subunit composition, substrate specificity and sensitivity to PI 3-kinase inhibitors (some of these features are summarised in the Table 1.2). Additionally, multiple isoforms of the p85 (α/β) regulatory and p110 (α, β, δ) catalyic subunits exist. Thus, functional diversity of the prototypical PtdIns 3-kinase-dependent pathway is offered by the following: (i) p110δ, unlike p110 α or β, does not phosphorylate the tightly associated p85 subunit, but instead harbours an autophosphorylatory capacity [Vanhaesebroeck *et al.* (1997)] which raises the possibility of differential patterns of protein phosphorylation occurring. (ii) compartmentalisation of the D-3 PtdIns lipids formed by different receptors or classes of PtdIns 3-kinases. (iii) distinct effector proteins mediated by individual D-3 PtdIns lipids formed by different receptors. (iv) multiple protein-protein interactions via the SH2/SH3/proline rich regions of p85.

1.5.3 PI 3-kinase couples to CD28 and the TCR.

PI 3-kinase couples to CD28 and CTLA-4 [Schneider *et al.* (1994)] apparently via a direct interaction between src-homology 2 (SH2) domain motifs and a (p)YXXM in the cytoplasmic tails of these molecules. The interaction of the YXXM motif with SH2 domains of the p85 subunit depends on the tyrosine phosphorylation of this motif. CD28 is tyrosine phosphorylated following ligation [Pages *et al.*(1994); August and DuPont (1994)], and mutagenesis [Pages *et al.* (1994); Prasad *et al.* (1994); Truitt *et al.* (1995)] and phosphopeptide binding studies [Prasad *et al.* (1994)] have confirmed that PI 3-kinase binds directly to CD28 via the (p)^{173}YMNM motif.

The coupling of PI 3-kinase to the TCR must occur independently of a (p)YXXM motif

since this motif is absent from the TCR. ITAMs (see section 1.2.1) however, are expressed singly in TCRγ, δ and ε chains, whilst the TCR ζ chain contains three (ζ1, ζ2 and ζ3). PI 3-kinase predominantly associates with phosphopeptides that correspond to the phosphorylated ζ1 proximal motif [Exley *et al.* (1994)] and has been detected in ζ immunoprecipitates [Carrera *et al.* (1994)]. Although both the TCR [Ward *et al.* (1992)] and CD28 [Ward *et al.* (1993)] activate PI 3-kinase as evidenced by the accumulation of D-3 phosphoinositide lipids, there is a considerable difference in the magnitude of responses elicited with CD28 inducing a response 5-10 fold greater than the TCR.

1.5.4 Regulation of PI 3-Kinase signal by an SH2 containing inositol 5' phosphatase (SHIP).

Myo-inositol containing phospholipids represent a minor, but metabolically highly active, component of the cell membrane playing a central role in signal transduction pathways. Enzymes which terminate the signal transduction processes and regulate the levels of inositol phosphate and phospholipid messengers are thus essential to proper cell function. This is graphically illustrated by the case of Lowe's oculocerebrorenal syndrome, where aberrant cell function is found to represent a mutation in an inositol polyphosphate 5'-phosphatase protein [Attree *et al.* (1992)]. A family of inositol 5'-phosphatases exists and these are summarised in Table 1.3. A recently identified inositol polyphosphate 5'-phosphatase that is expressed in haematopoietic cells is SHIP [Damen *et al.* (1996)]. SHIP is a 145 kDa protein consisting of an inositol polyphosphate 5'-phosphatase catalytic domain, an NH_2 terminal SH2 domain, a COOH terminal proline rich domain, which may bind SH3 domains mediating protein-protein interactions, and two NPXY motifs which when phosphorylated, act as ligands for phosphotyrosine binding domains [Tridandapani *et al.*(1997)]. The structure of SHIP is represented schematically in Figure 1.6.

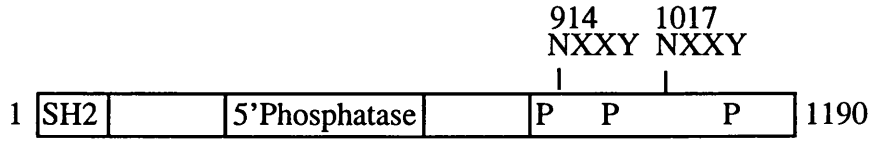


Figure 1.6 Schematic representation of the structure of SHIP. Amino acid sequence of SHIP isolated from murine haematopoietic cell line $B6SUtA_1$ is given in [Damen *et al.* (1995)]. SH2: Src-homology domain 2; 5' Phosphatase: inositol polyphosphate-5'-phosphatase domain; NXXY: potential protein tyrosine binding domain ligands; P: potential SH3 Ligands.

SHIP is tyrosine phosphorylated in a variety of activated haematopoietic cell lines, but not quiescent cells, in response to growth factors, cytokines and crosslinking of antigen receptors, possibly with *lck* playing a role [Lamkin *et al.* (1997)]. This tyrosine phosphorylation of SHIP mediates interactions with adaptor molecules such as Shc and Grb2 [Kavanaugh *et al.* (1996)], which are believed to mediate the subcellular location of the phosphatase. SHIP is unique, amongst 5-phosphatases, in that it targets only substrates that are phosphorylated on the D-3 position such as Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3.

5-phosphatase	MW	Substrates	Comments
5-phosphatase I	43 kDa	Ins(1,4,5)P_3 Ins(1,3,4,5)P_4	[Matzaris *et al.* (1994)]
5-phosphatase II	75 kDa	Ins(1,4,5)P_3 Ins(1,3,4,5)P_4 PtdIns(4,5)P_2 PtdIns(3,4,5)P_3	[Ross *et al.* (1991)]
OCRL	75 kDa	PtdIns(4,5)P_2 Ins(1,3,4,5)P_4 Ins(1,4,5)P_3	Golgi localised [Attree *et al.* (1992)].
Synaptojanin	145 kDa	Ins(1,4,5)P_3 Ins(1,3,4,5)P_4 PtdIns(4,5)P_2	Nerve terminal protein. Contains region homologous to yeast*Sac 1* gene which is implicated in phosphoinositide metabolism. [McPherson *et al.* (1996)]
SHIP	145 kDa	Ins(1,3,4,5)P_4 PtdIns(3,4,5)P_3	Unique in substrate specificity and SH2 domain [Damen *et al.* (1996)]
SHIP2	142 kDa	Ins(1,3,4,5)P_4 PtdIns(3,4,5)P_3	[Pesesse *et al.* (1998)]
INPPL-1	126 kDa	Not demonstrated to date.	Closely homologous to SHIP. Discovered in Fanconi's anaemia cells. [Lioubin *et al.* (1996)]

Table 1.3: Summary of known 5-phosphatases. All enzymes mentioned encode a signature motif of 5-phosphatases comprising GDXN(F/Y)R and /or P(A/S)W(C/T)DRIL, however alignment of family members indicates the catalytic domain spreads over 300 amino acids with several highly conserved motifs [Mitchell *et al.* (1996)]. Additionally, inositol polyphosphate-like protein 1 (INPPL 1), SHIP and Synaptojanin contain carboxy terminal proline rich regions, which bind to SH3 domains.

SHIP has been implicated as inhibitory to B cell activation [Amigorena *et al.* (1992)], due to its association with FcγRIIB (CD32), a low affinity IgG receptor expressed on monocytes and B cells. IgG antibodies inhibit B cell activation by co-aggregating FcγRIIB and the B cell antigen receptor. The negative regulatory function of FcγRIIB was found to be dependent upon the presence of an I/VxYxxL/V motif, termed the immunoreceptor tyrosine-based inhibitory motif (ITIM) [D'ambrosia *et al.* (1995)], reflecting its structural and functional similarity to the ITAM (see section 1.2). FcγRIIB has also been found to inhibit cells activated by single chain chimaeric molecules bearing the intracytoplasmic domain of Ig-α [Muta *et al.* (1994)], FcRγ or CD3 ζ [Daeron *et al.* (1995)]. Thus, rather than being a B cell specific regulatory molecule, FcγRIIB is a general negative co-receptor for all ITAM-bearing receptors expressed by haematopoietic cells. The mechanisms of SHIP inhibitory signalling in B cell activation are obscure but may involve modulating calcium influx [Muta *et al.* (1994); Ono *et al.* (1996)], via an effect on Bruton's tyrosine kinase (Btk) [Bolland *et al.* (1998)]. PtdIns(3,4,5)P_3 in turn recruits Btk to the plasma membrane through an interaction with its PH domain, to regulate calcium fluxes. Activation of SHIP inhibits the levels of PtdIns(3,4,5)P_3 inhibiting Btk association with the plasma membrane. Whether SHIP truly represents an "off" switch, or rather a modification of the cell activating signal is a moot point. Taking the case of PI 3-kinase generated PtdIns(3,4,5)P_3, clearly SHIP activity will deplete levels of the putative signalling molecule. However, there may be downstream targets for the SHIP-generated inositides, that include PtdIns(3,4)P_2, such as PKB [Carpenter and Cantley (1996)] or PKC family members [Toker *et al.*(1994)].

1.5.5.1 Inhibitors of PI 3-kinase: Wortmannin.

Whilst the exact role of the D-3 phosphorylated products of PI 3-kinase is not entirely clear, one of the most valuable tools in elucidating a role for PI 3-kinase is wortmannin [Arcaro and Wymann (1993)]. Wortmannin is a cell permeant, fungal metabolite which has been widely employed as an inhibitor of PI 3-kinase-dependent pathways, since PI 3-kinase enzymatic function was shown to be inhibited by wortmannin at low nanomolar concentrations (e.g. <10 nM) [Arcaro and Wymann (1993)]. Inhibition of both protein serine and lipid kinase activities is achieved by covalent binding to the p110 catalytic subunit [Thelen *et al.* (1994)], an interaction which appears dependent upon Lys^{802} within p110 [Wymann *et al.* (1996)]. Some of the cellular functions inhibited by wortmannin are summarised in Table 1.4.

Functional response	Agonist	Wortmannin concentration (nM)	Reference
i) Respiratory burst from neutrophils	fMLP	5	Arcaro and Wymann (1993)
ii) T cell chemotaxis	RANTES	5	Turner *et al.* (1996)
iii) IL-2 production from T cells	CD28	10	Ward *et al.* (1995)
iv) T cell proliferation	CD28+CD3	10-100	Karnitz *et al.* (1995)
v) Actin rearrangements in fibroblasts	PDGF	5	Wymann and Arcaro (1994)
vi) Membrane ruffling in endothelial cells	PDGF	10-100	Wennstrom *et al.* (1994)
vii) β1 integrin upregulation in HL60 cells transfected with CD2/CD2$^+$ T cells	Antibody stimulation of CD2	10-100	Shimizu *et al.* (1995)
viii) Histamine secretion and leukotriene release from RBL cells	FcεR1	3	Yano *et al.* (1993)
ix) Phagocytosis in U937 cells	Fcγ receptor induced	10-100	Ninomiya *et al.*(1994)
x) Inhibition of iNOS		10-100	Wright *et al.* (1997).

Table 1.4 Functional responses of PI 3-kinase. Some of the functional responses sensitive to nanomolar concentrations of wortmannin are given in the Table.

Limitations exist however on the use of wortmannin as a specific inhibitor of PI 3-kinase, since further targets for wortmannin have been identified in phospholipase A_2 (with an IC_{50} of 2 nM) [Cross *et al.* (1995)] and mammalian soluble PI 4-kinase (with an IC_{50} of 3-5 nM) [Nakanishi *et al.* (1995)], and at elevated concentrations phospholipase D

[Reinhold *et al.* (1990)] and phospholipase C [Bonser *et al.* (1991)]. Additionally wortmanin has been reported to inhibit the mammalian target of rapamycin (mTOR) which regulates p70 S6 kinase, a potential PI 3-kinase target (see section 1.5.6.2) [Brunn *et al.* (1996)]. Further problems are placed on the interpretation of data generated using wortmannin as it inhibits different classes of PI 3-kinases to different extents, for example wortmannin inhibits class IA PI 3-kinases and class III PI 3-kinase with an IC_{50} of <10 nM [Arcaro and Wymann (1993); Volinia *et al.* (1995)], whilst the IC_{50} for inhibition of class IB PI 3-kinase is 40-50 nM. Furthermore, mammalian class II PI 3-kinases such as PI 3-kinase C2α are refractory to wortmannin [Domin *et al.* (1997)].

1.5.5.2 Other inhibitors of PI 3-kinase.

LY294002 Wortmannin

Figure 1.7 The chemical structures of LY294002 and wortmannin. Wortmannin [Arcaro and Wyman (1993)] and LY294002 [Vlahos *et al.* (1994)] are structurally unrelated, but both inhibit PI 3kinase with an IC_{50} of 5nM and 1.4 mM, respectively.

A novel PI 3-kinase inhibitor, namely 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) has been synthesised, and demonstrated to inhibit purified bovine brain PI 3-kinase with an IC_{50} of 1.4 μM [Vlahos *et al.* (1994)]. LY294002 was found to inhibit PI 3-kinase dependent proliferation of cultured rabbit aortic smooth muscle cells and completely abolish fMLP-induced PI 3-kinase activity in human neutrophils. No significant effects of LY294002 were observed on the activity of other enzymes including PI 4-kinase, src, MAP-kinase, S6 kinase, DAG kinase, PKA and PKC [Vlahos *et al.* (1994)].

Demethoxyviridine is structurally related to, but distinct from, wortmannin and has been observed to inhibit purified class-IA PI 3-kinase with an IC_{50} of 3.4 nM [Wolscholki *et al.* (1994)]. Demethoxyviridine, in common with wortmannin however, has been reported to inhibit targets other than PI 3-kinase [Bonser *et al.* (1991)].

1.5.5.3 Alternative strategies to investigate the role of PI 3-kinase.

The potential problems of non-specific inhibitors can be circumvented by the use of mutated PI 3-kinase isoforms. Several cDNAs corresponding to PI 3-kinase have been engineered to produce active or inactive forms, to demonstrate either an effect of increased PI 3-kinase activity or an effect of blocking PI 3-kinase by using a dominant negative type approach. Mutated forms of p85 that lack the p110-binding site, have been shown to behave as dominant negative proteins that block activation of PI 3-kinase activity [Hara *et al.* (1994)], presumably by binding through their SH2 domains and forming non-productive complexes. Such a dominant negative mutant has previously been observed to inhibit insulin-stimulated PtdIns(3,4,5)P_3 accumulation in U937 cells [Stephens *et al.* (1995)]. An antisense cDNA vector to p85 has also been used to block class IA PI 3-kinase activation [Yin *et al.* (1998)]. Alternatively, a form of p110 covalently attached to the inter SH2 domain (p110 binding domain) of p85, via a glycine linker region, has been shown to be active independently of p85-mediated regulation [Hu *et al.* (1995)]. Genetic approaches have also demonstrated membrane localisation of the p110 subunit by myristoylation, farnesylation [Klippel *et al.* (1996)], or by fusing the catalytic domain of p110 with the extracellular domain and transmembrane region of rat CD2 [Reif *et al.* (1996)], as sufficient to result in a constitutively active PI 3-kinase mutant. Generally, the combined use of PI 3-kinase inhibitors and blocking or activation of PI 3-kinase using molecular reagents, allows for the construction of more robust arguments regarding the role of the enzyme in specific cellular events being studied.

1.5.6 PI 3-kinase dependent signalling cascades

The biochemical events downstream of PI 3-kinase are currently the subject of intense research. Recently, it has become increasingly apparent that PI 3-kinase may mediate its effect by the interaction of certain D-3 phosphoinositide lipids with pleckstrin homology (PH) domains. PH domains are protein modules of around 100 amino acids found in several proteins involved in signal transduction [Haslam *et al.* (1993)]. Although its specific function has yet to be elucidated, the carboxy terminal regions of many PH domains bind to the $\beta\gamma$ subunits of G proteins [Koch *et al.* (1993)]. A further clue to the function of PH domains lies in their ability to bind phosphoinositide lipids [Haslam *et al.* (1993); Harlan *et al.* (1994)], which may facilitate the localisation of the PH domain containing protein to phospholipid membranes. Indeed, many PH domain containing

proteins need to be associated with membranes for their function, but lack classical membrane-anchoring groups such as a hydrophobic helix or sites for post-translational addition of lipid molecules. For example, βG-spectrin, which contains a PH domain, has been shown to interact with brain membranes depleted of peripheral proteins [Davis and Bennett (1994)], but does not contain a classical membrane-anchoring group. Many of the reported downstream effectors of PI 3-kinase contain PH domains and these include PKB [Klippel *et al.* (1997)], PLCγ1 [Falasca *et al.* (1998)], tyrosine kinases such as ITK [August *et al.* (1997)] and Bruton's tyrosine kinase [Salim *et al.* (1996)], and the guanine nucleotide exchange factor Vav [Han *et al.* (1998)]. Of the growing number of putative downstream targets of PI 3-kinase, a number are particularly relevant to T cell activation, and are discussed below.

1.5.6.1.1 Protein kinase B (PKB)

Protein kinase B was identified independently in 1991 by three groups, leading to a potentially confusing nomenclature of the molecule as protein kinase B [Coffer *et al.* (1991)], RAC-protein kinase [Jones *et al.* (1991)] or Akt [Bellacossa *et al.*(1991)]. To avoid confusion the molecule is referred to herein as protein kinase B (PKB). The molecule is approximately 60 kDa consisting of a serine/threonine kinase domain stretching from residues 148-411, which displays 73% homology with the kinase domain of PKC ε, and 68% homology with the kinase domain of protein kinase A. Additional to the catalytic domain, there is a pleckstrin homolgy domain (PH) at the amino terminal end, which makes up the major part of the amino-terminal regulatory domain spanning residues 1-147, and has been referred to as the Akt homology (AH) domain. The carboxy-terminal tail region accounts for the rest of the protein, see Figure 1.8 below.

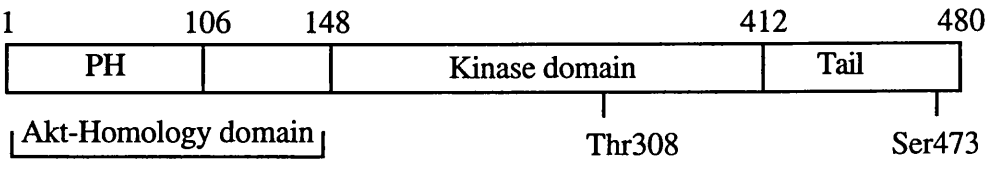


Figure 1.8 Diagrammatic structure of human PKBα.

1.5.6.1.2 Regulation of PKB.

PKB is activated in cells in response to a variety of growth stimuli including platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin, thrombin and nerve growth factor (NGF). A number of lines of evidence suggest a role for PI 3-kinase in the regulation of PKB: (i) Growth factor induced activation of PKB is inhibited by wortmannin [Franke *et al.* (1995)], and expression of a dominant negative form of p85 [Burgering and Coffer (1995)]; (ii) PDGF receptor mutants which fail to activate PI 3-kinase, also fail to activate PKB [Burgering and Coffer (1995)]; and (iii) active mutants of PI 3-kinase activate PKB. Indeed, a wealth of publications have implicated PKB as an important target for PI 3-kinase activity.

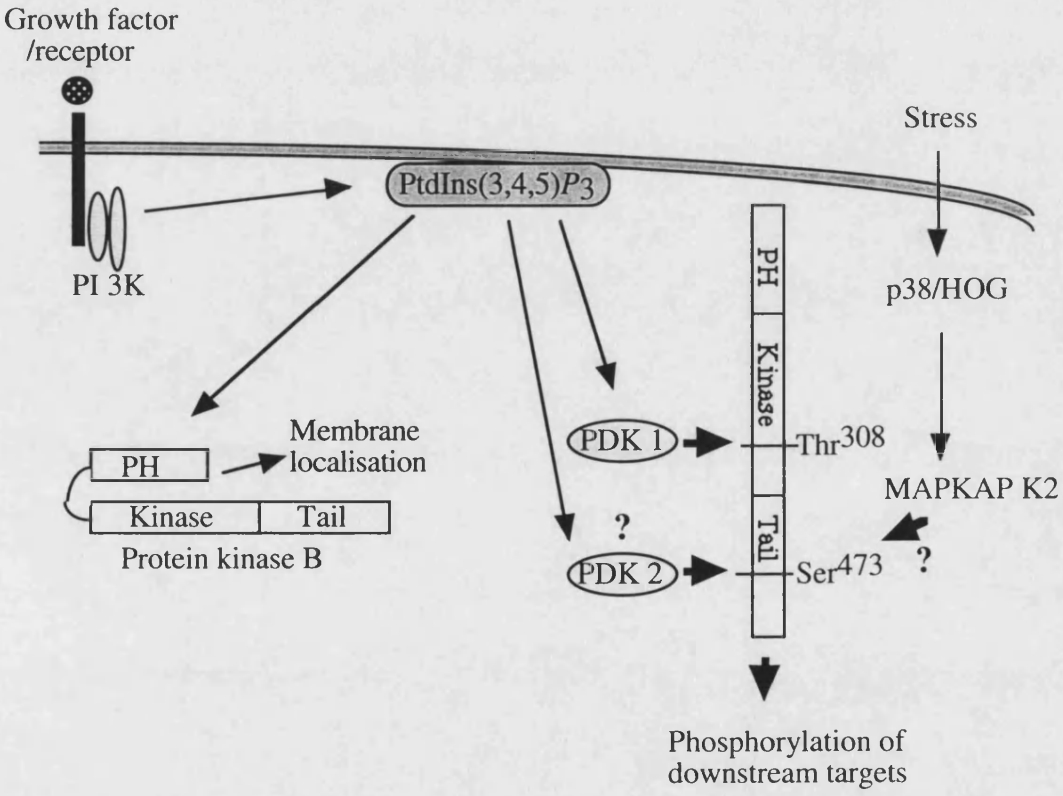


Figure 1.9 Schematic model for activation of protein kinase B. Following growth factor receptor stimulation, PI 3-kinase is activated generating PtdIns(3,4,5)P_3, hydrolysis of this lipid by 5-phosphatases leads to PtdIns(3,4)P_2 formation. Both lipids interact with the PH domains of PKB and PDK1 inducing co-localisation at the plasma membrane where PKB is subject to phoshorylation by PDK1 at ^{308}Thr and the putative PDK2 at ^{473}Ser, inducing full activation. Stress activation of the p38 MAP kinase pathway represents a potential route for PKB activation since MAPKAP K2 has been demonstrated to phosphorylate PKB at ^{473}Ser at least *in vitro* [Alessi *et al.* (1996)].

PKB is activated synergistically by the dual phosphorylation of its ^{473}Ser and ^{308}Thr residues [Alessi *et al.* (1996)]. The amino acid sequences around these sites are very different, suggesting that two distinct kinases catalyse these phosphorylations. One PKB directed kinase has been identified in PtdIns(3,4,5)P_3-dependent kinase 1 (PDK1) which phosphorylates ^{308}Thr [Alessi *et al.* (1997)]. The ^{473}Ser-kinase has been provisionally termed PDK2 by Alessi *et al.* although the molecule has not been cloned or purified to date. PDK2 also appears to be dependent on PI 3-kinase activity since phosphorylation of ^{473}Ser, in response to insulin, is inhibited by wortmannin [Alessi *et al.* (1996)].

PKB binds both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 via its PH domain, although the interaction with PtdIns(3,4)P_2 is about ten-fold weaker [James *et al.* (1996); Frech *et al.* (1997)]. Neither lipid directly affects the activity of PKB [James *et al.* (1996); Alessi *et al.* (1997)], however, PKB is translocated to the plasma membrane as a result of lipid binding. PDK1 also binds both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 via its PH domain, and is consequently translocated to the plasma membrane [Anderson et al. (1998)]. Similar to PKB, the kinase activity of PDK1 is unlikely to be affected by the binding of lipids since PDK1 activity is not stimulated by insulin *in vitro* [Alessi *et al.* (1997)]. Furthermore, the recently reported ability of PDK1 to phosphorylate p70 S6 kinase is not regulated by PtdIns(3,4,5)P_3 [Pullen *et al.* (1998)]. The ability of the D-3 phosphoinositides to "activate" PDK1 may result from a substrate concentrating effect, and/or the induction of a conformational change in PKB that facilitates the phosphorylation of ^{308}Thr. After phosphorylation at both sites, activated PKB may detach from the membrane to phosphorylate cytosolic targets. Evidence also exists for the nuclear translocation of PKB isoforms within 20-30 minutes following stimulation of cells [Meier et al. (1997)].

Discrepancies from this model have arisen in the literature, suggesting that PtdIns(3,4)P_2 may have a direct effect upon the activity of PKB [Frech *et al.* (1996); Franke *et al.* (1997); Klippel *et al.* (1997)], and that whilst PtdIns(3,4)P_2 is stimulatory, PtdIns(3,4,5)P_3 may be inhibitory to PKB activity [Frech *et al.* (1996); Franke *et al.* (1997)]. These observations may be explained by PDK1 contamination of the PKB preparations, and inhibitory contaminants of synthetic phospholipids respectively.

In addition to PI 3-kinase-dependent PKB regulation, further pathways must clearly exist since cellular stresses, such as heat shock and hyperosmolarity (both of which activate the p38/HOG1 cascade) can stimulate the PKB activity [Konishi *et al.* (1994)]. Mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAP kinase 2), a direct target of p38/HOG1, can phosphorylate PKB at least *in vitro* [Alessi *et al.* (1996)] and

may represent a potential PKB activating pathway for cellular stresses by mechanisms independent of PI 3-kinase. A model of PKB activation representing these regulatory inputs is shown in Figure 1.9.

1.5.6.1.3 Downstream targets of PKB.

The downstream targets of PKB are currently the subject of keen interest since activation of the kinase has been shown to suppress the apoptotic death of a number of cell types in response to U.V. radiation [Kulik *et al.* (1997)], withdrawal of insulin like growth factor-1 (IGF-1) from neuronal cells [Dudek *et al.* (1997)] and detachment of cells from an extracellular matrix (anoikis) [Kwhaja *et al.* (1997)]. A direct substrate for PKB has been identified in glycogen synthase kinase 3 (GSK 3) [Cross *et al.* (1995]. Phosphorylation of GSK 3 by PKB, results in its inactivation and subsequent activation of glycogen synthesis [Cross *et al.* (1995)]. A further target for PKB is the ribosomal protein S6 kinase $p70^{S6K}$, although there is no evidence to suggest that this is a direct substrate [Burgering and Coffer (1995)]. Additionally PKB has been shown to stimulate glucose uptake and GLUT 4 translocation [Kohn *et al.* (1996)]. Recently a further target for PKB has been described, namely BAD, which is more relevant to the cell survival mechanism of PKB. BAD is a distant member of the Bcl 2 family of proteins (see section 1.7) which, when unphosphorylated, interacts and inactivates the cell survival factors Bcl-X_L and Bcl-2, and can thus promote apoptosis [Zha *et al.* (1996)]. PKB can phosphorylate a critical serine residue, ^{136}Ser , on BAD causing it to dissociate from, and thus activate the cell survival factor Bcl X_L [Datta *et al.* (1997)]. The serine phosphorylated BAD is then bound preferentially to a cytosolic protein termed 14-3-3 [Zha *et al.* (1996)] and this may represent a specific mechanism for sequestration of BAD during cell survival.

1.5.6.2 p70 S6 kinase

A further target for PI 3-kinase is p70 S6 kinase, a ubiquitous, mitogen activated serine/threonine kinase, that is necessary for G1 to S-phase progression within the cell division cycle [Reinhard *et al.* (1994)]. On activation, the enzyme is subject to multi-site phosphorylation and in turn phosphorylates the 40S ribosomal subunit protein S6, which correlates with increased translation, especially of mRNAs containing a polypyrimidine tract in their 5' untranslated regions [Jefferies *et al.* (1997)]. Essentially two lines of evidence suggest that p70 S6 kinase is a downstream effector of PI 3-kinase. Firstly, PI 3-kinase is required for the phosphorylation of ^{252}Thr in the catalytic domain of p70 S6

kinase [Weng *et al.* (1995)], and secondly, activation of the enzyme by insulin or PDGF is sensitive to wortmannin [Chung *et al.* (1994)]. The mechanism of PI 3-kinase activation of p70 S6 kinase however, is unclear and in fact one study has suggested the effect of wortmannin in blocking p70 S6 kinase activation may not be due to inhibition of PI 3-kinase, since a dominant negative form of PI 3-kinase that blocked insulin stimulated PI 3-kinase activity, was unable to block activation of p70 S6 kinase [Hara *et al.* (1995)]. Studies of p70 S6 kinase regulation involving wortmannin may have been complicated by the fact that the genes encoding the targets of rapamycin in yeast TOR 1 and TOR 2 [Kunz *et al.* (1993)], and the human homologue mammalian target of rapamycin (mTOR) or FKBP-rapamycin associated protein (FRAP), which are upstream regulatory proteins of p70 S6 kinase, show strong homology with domains of p110. Lipid kinase enzymatic activity of the TOR proteins has not been demonstrated, but the kinase domain has been shown to be important for their G1 cell cycle function [Zheng *et al.* (1995)]. Furthermore, wortmannin and LY294002 have been demonstrated to inhibit autokinase activity of mTOR [Brunn *et al.* (1996)]. Therefore, it is possible that apparent effects of PI 3-kinase on p70 S6 kinase activity may be due to reported sequence homology between mTOR and PI 3-kinase.

Two studies have also suggested the recently cloned 3-phosphoinositide-dependent kinase 1 (PDK1) is an upstream p70 S6 kinase regulatory molecule [Alessi *et al.* (1997b); Pullen *et al.* (1998)]. PDK 1 was isolated and cloned on the basis of its ability to phosphorylate PKB [Alessi *et al.* (1997a)]. Using purified recombinant forms of PDK 1 and p70 S6 kinase, it has been demonstrated that PDK 1 specifically phosphorylates p70 S6 kinase at residue ^{229}Thr *in vitro* [Alessi *et al.* (1997b); Pullen *et al.* (1998)]. Although PKB and p70 S6 kinase differ structurally, their sequences show that ^{229}Thr and ^{389}Thr of p70 S6 kinase are analogous to ^{308}Thr and ^{473}Ser of PKB respectively [Pullen *et al.* (1998)], and this may reflect a regulatory mechanism conserved for PKB and p70 S6 kinase [Reviewed by Downward (1998)]. The curent understanding of p70 S6 kinase activation [Reviewed more comprehensively by Pullen and Thomas (1997)] can be summarised in the following model: Proline directed kinases (such as MAP kinases) phosphorylate a number of sites in the COOH auto-inhibitory domain [Ferrari *et al.* (1992)]. This relieves a conformational inhibition that may result from the interaction of the NH_2 and COOH terminals of the protein. ^{389}Thr is then phosphorylated by an unidentified PtdIns(3,4,5)P_3-dependent kinase, suggested as the putative PDK 2 [Pullen *et al.* (1998)]. Both of these steps are required to expose ^{229}Thr as a substrate for the constitutively active PDK 1, yielding active p70 S6 kinase. The multisite phosphorylation of p70 S6 kinase is suggestive of a complex control mechanism, however, clarification of the regulation of S6 kinase may be an important step in understanding how cell

proliferation is controlled.

1.5.6.3 Mitogen activated protein kinase cascades.

Mitogen activated protein kinase (MAP kinase) was initially identified as a serine/threonine kinase, based on its ability to phosphorylate microtubule-associated protein [Ray and Sturgill (1987)]. Much investigation has revealed the MAP kinase family as a cascade of proteins, representing a major intracellular signalling system. MAP kinases are activated by a diverse array of extracellular stimuli and regulate a variety of cellular processes. Although, in mammalian cells, a degree of cross-talk exists, three cascades have been elucidated in the extracellular regulated kinases (ERKs), the c-jun NH_2-terminal kinases (JNKs or stress activated protein kinases) and p38. These three cascades are represented schematically in the Figure 1.10.

Evidence is accumulating that PI 3-kinase may be involved in the regulation of either or both of the signalling cascades that control ERK or JNK activity, and thus regulation of c-jun or c-fos. This is supported by the observation that fos transcription can be activated by a constitutively active p110 mutant [Hu *et al* (1995)]. Since this is a Ras dependent effect it implies that PI 3-kinase can modulate the $p21^{ras}$ pathway, and thus, the eventual modulation of ERKs that are thought to be involved in the transmission of signals from $p21^{ras}$ to the nucleus. Furthermore, the ras-related GTP-binding protein rac, which has been implicated as an upstream regulator of JNK [Minden *et al.* (1995)], appears to be a downstream effector for PI 3-kinase.

Interestingly JNK has been reported as synergistically activated by CD28 and TCR ligation in the leukaemic T cell line Jurkat [Su *et al.* (1994)]. This has led to the suggestion that JNK represents a point of integration of the signals for T cell activation and IL-2 production.

Similarly, a recent report also implicated p21-CDC42/Rac as a point of integration of the CD3 and CD28 signals [Kaga *et al.* (1998)]. Activation of PAK may be mediated via ceramide generation since treatment of Jurkat cells with C2-ceramide could mimic the activating effect of CD28 [Kaga *et al.* (1998)], correlating well with previous data [Westwick *et al.* (1995)], thus this pathway could potentially be independent of PI 3-kinase. The importance of this pathway has been demonstrated by the use of murine T cells deficient in the expression of SEK1, which show impaired CD28 mediated IL-2 production and proliferation [Nishina *et al.* (1997)].

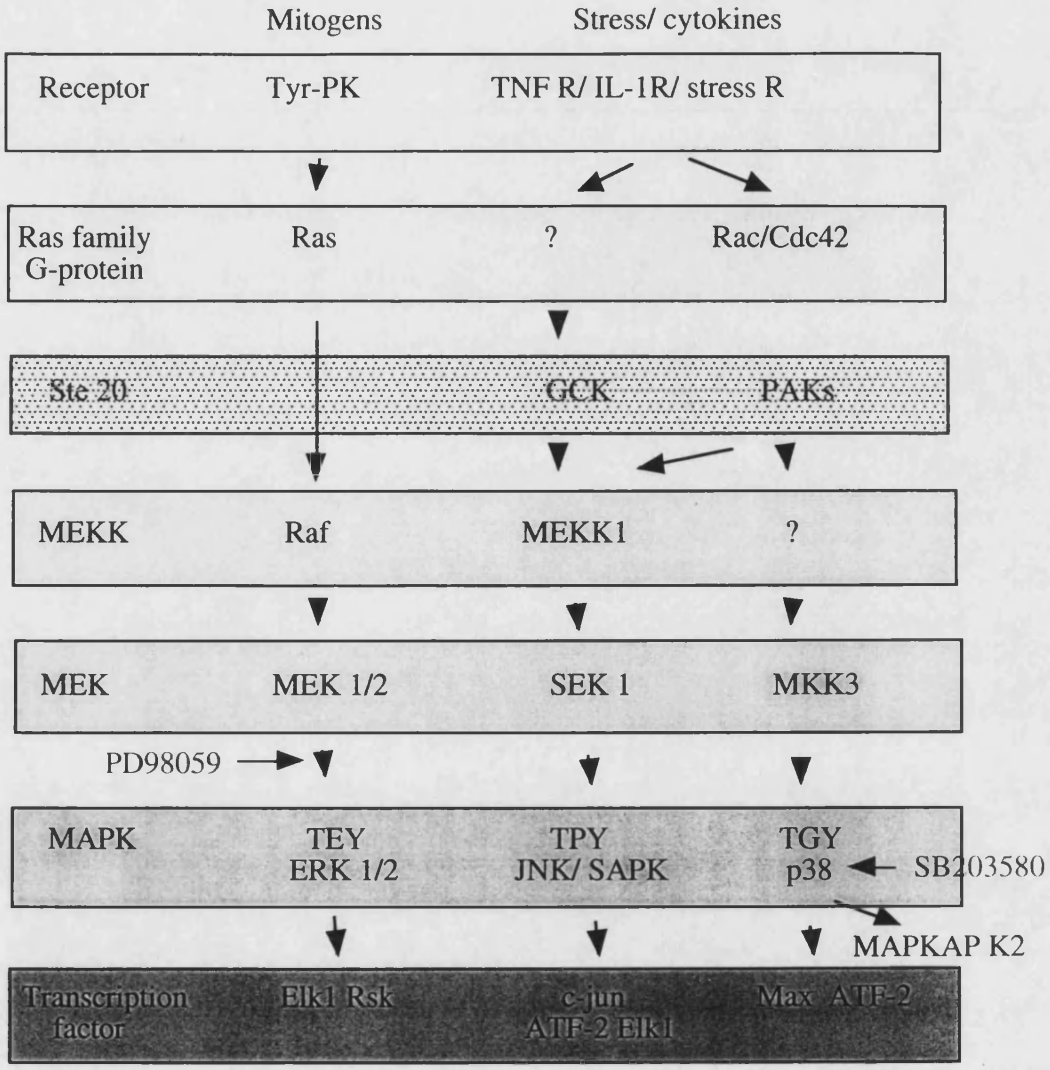


Figure 1.10 Schematic summary of the mitogen activated protein kinases. [Adapted from Kyriakis and Avruch (1996)]. Although represented here as linear, a degree of cross-talk does occur within mammalian cells. TEY, TPY and TGY are amino acid sequences characteristic to each pathway that are dually phosphorylated as a requirement for MAP kinase activation. SB203580 and PD98059 are pharmacological inhibitors. Activation of ERKs is induced by agonists for tyrosine kinase coupled receptors such as EGF, PDGF and TCR/CD3 complex can involve ras-mediated translocation to the plasma membrane, of the upstream kinase c-raf, which phosphorylates, thus activating the downstream kinase MAPK/ERK kinase (MEK, also known as MAPK kinase 1, MKK1). The JNK family of MAP kinases are activated by cellular stresses (such as U.V. light, γ-irradiation and osmotic or heat stress), inflammatory cytokines (TNFα and interleukin-1) and ceramides. JNKs phosphorylate the NH_2-terminal activation domain of c-jun and ATF 2, increasing their transcriptional activity. p38 MAP kinase is activated by osmotic stress and lipopolysaccharide (LPS). Substrates of p38 include MAPKAP kinase 2 [Pulverer *et al.* (1991)], and the ATF 2 transcription factor. PAK 1 (p21 activated kinase) is activated directly on binding active Rac 1 and Cdc 42 *in vitro*. GCK is germinal center kinase [Katz *et al.* (1994)]. Max is a Myc binding protein phosphorylated *in vitro* by Mxi 2 (a p38 isoform) [Zervos *et al.* (1995)].

1.5.6.4 PI 3-kinase regulates the phospholipase C pathway.

A large variety of extracellular signals stimulate the hydrolysis of PtdIns(4,5)P_2 by the activation of PLC isoforms [Rhee and Bae (1997)]. The two products of this hydrolysis, Ins(1,4,5)P_3 and diacylglycerol, mediate the release of intracellular calcium [Berridge (1993)], and activation of protein kinase C [Nishizuka (1995)], respectively. The critical role of these pathways in mammalian growth and development is demonstrated by the recent finding that targeted deletion of PLCγ1 causes embryonic lethality in mice [Ji *et al.* (1997)]. It has been demonstrated that phosphorylation of ^{783}Tyr of PLCγ1 is essential for stimulation of enzymatic activity in NIH-3T3 cells stimulated with PDGF [Kim *et al.* (1991)]. However tyrosine phosphorylation does not strictly correlate with Ins(1,4,5)P_3 production, since some extracellular signals induce weak tyrosine phosphorylation of PLCγ1 and extensive production of Ins(1,4,5)P_3, whilst other signals cause strong tyrosine phosphorylation of PLCγ1, yet only low levels of Ins(1,4,5)P_3 production [Rhee and Bae (1997)]. Therefore, additional control mechanisms, independent of PLCγ1 tyrosine phosphorylation are likely to play a role in PLC enzymatic activity. Indeed, recent publications have suggested a role for PtdIns(3,4,5)P_3 in the activation of PLCγ1 via interactions of the lipid with both the PH domain [Falasca *et al.* (1998)], and the SH2 domain [Rameh *et al.* (1995); Bae *et al.* (1998)] of PLCγ1, and this may represent an important means of regulation.

In common with the TCR, CD28 has been reported to regulate the tyrosine phosphorylation of PLCγ1, PtdIns hydrolysis and the cytosolic free calcium concentration [Ledbetter *et al.* (1986); Weiss *et al.* (1986)]. Discrepancies appear however in the relative ability of CD28 to elicit these responses, dependant upon the type of T cell model used, for example, in Jurkat cells, PtdIns hydrolysis, increases in intracellular calcium and diacyglycerol generation can be elicited in the absence of cross linking [Nunes *et al.* (1993)]. However, it has been noted that the ability of anti-CD28 mAbs to elicit these signals is dependent on the epitope recognised by the mAb [Nunes *et al.* (1993)]. Moreover, other groups have reported that CD28 cross-linking is a prerequisite for eliciting PLC activity in purified resting T cells, activated T cells and human CD28-transfected murine T cell hybridoma [Ledbetter *et al.* (1986); Weiss *et al.* (1986); Ledbetter *et al.* (1990); Couez *et al.* (1994)]. In marked contrast to anti-CD28 mAbs, B7.1 failed to elicit any detectable activation of PLC as assessed by PtdIns(4,5)P_2 degradation and phosphatidic acid generation [Ward *et al.* (1993)]. This suggests that PLC activation following mAb stimulation of CD28 may be functionally redundant.

1.5.6.5 PI 3-kinase activates protein kinase C (PKC) family isoforms.

Members of the serine/threonine protein kinase family, protein kinase C have been shown to be activated by the second messenger diacylglycerol (DAG), which is formed following agonist-dependent turnover of inositol lipids. This is known to form the basis of a signalling pathway [Hokin (1985)]. However, the present family of PKC isozymes contains 11 members that differ in their structure and function, observations which form the basis of their grouping: Conventional PKC's α, β and γ; Novel PKC's δ, ε, η and θ; Atypical ζ, λ and ι, and PKC μ. Most PKC isotypes (with the exception of atypical isoforms) display DAG, or phorbol ester, dependence for activation. The family of PKC isozymes is summarised in Table 1.5. The PKC isozymes δ, ε, η and ζ have been shown to be activated, at least *in vitro*, by the lipid products of PI 3-kinase [Nakanishi *et al.* (1993); Toker *et al.* (1994)]. Palmer *et al.* (1995) suggested however, that this may be an *in vitro* artifact resulting from lipid vesicles in the assays that contained highly charged phospholipids. On comparing the *in vitro* activity of PKCβ, ε, η, ζ and a member of the PKC-related kinase family, PRK1, both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 activated each of the enzymes with similar activation constants, implying a lack of specificity in this *in vitro* analysis [Palmer *et al.* (1995)]. A few studies however, have linked the activation of PI 3-kinase to the activation of protein kinase C isoforms in whole cells. For example, the addition of PtdIns(3,4,5)P_3 to permeabilised platelets, stimulated the phosphorylation of pleckstrin, a major PKC substrate, and the lipid could bypass the block of PI 3-kinase activity by wortmannin [Zhang *et al.* (1995)]. Other studies have also demonstrated that insulin mediated activation of PKCζ can be blocked by PI 3-kinase inhibitors and expression of dominant negative p85 [Standaert *et al.* (1997)].

Sub group	Isoforms	DAG	PdBu	Ca^{2+}
conventional	α, β, γ	+	+	+
novel	δ, ε, η, θ	+	+	-
atypical	ζ, λ, ι	-	-	-
PKC μ	μ	+	+	-

Table 1.5 The protein kinase C family. The 11 members of the present family of PKC enzymes are grouped on a combination of structural and functional considerations. Sensitivity to diacylglycerol (DAG), Phorbol dibutyrate (PdBu), or calcium are indicated. PKC μ is also referred to as protein kinase D [Van Lint *et al.* (1995)].

Additional to lipid mediated regulation of PKC, two groups have shown a direct association between PI 3-kinase and PKC isoforms, such as PKCδ and PKCε in human

haematopoietic cells and platelets [Ettinger *et al.* (1996)], and PKCζ during IL-2 stimulation of murine T cells [Gomez *et al.* (1996)]. This could enable the direct phosphorylation of PKC by PI 3-kinase, allowing for further complexity in PKC regulation.

Studies with phorbol esters have previously implicated PKC in the control of many signalling pathways including those mediated by $p21^{ras}$ and the mitogen activated protein (MAP) kinases such as extra cellular signal-regulated kinases (ERK) and c-jun N-terminal kinase (JNK). The PKC responsive element in the enhancer of the IL-2 gene contains sites for NF-κB, AP-1 and NF-AT1 [Genot *et al.* (1995)]. Although the role of specific isoforms is poorly understood, PKCε has been implicated in the induction of AP-1 and NF-AT1 [Genot *et al.* (1995)], whilst PKCζ has been suggested to play a role in NF-κB induction in fibroblasts (although not in T cells, to date). Interestingly, PKCζ has also been suggested as a direct target for ceramide and, thus may play a role in sphingomyelinase signalling. Thus PKCζ could represent a potential target for integration of the CD28-induced PI 3-kinase signals and sphingomyelinase signals.

1.5.7.1 Other signalling pathways activated by CD28: Protein tyrosine phosphorylation.

Analysis of the cytoplasmic domain of CD28 reveals no obvious inherent enzymatic activity, however ligation is followed by tyrosine phosphorylation of a number of substrates including CD28 itself, Vav and the adaptor protein $p62^{dok}$ [Klasen *et al.* (1998)]. Thus in common with the TCR, CD28 must recruit non-receptor tyrosine kinases. Extending this analogy, it was further proposed that *src* family tyrosine kinases such as $p59^{fyn}$ and $p56^{lck}$ were coupled to CD28 [Prasad *et al.* (1994)]. Indeed, $p56^{lck}$ has been reported to be activated in Jurkat cells following CD28 ligation by cross linked antibody, but not by CD28 mAb alone [August and Dupont (1994)]. In *lck* deficient cell lines CD28 ligation by mAb elicits impaired calcium mobilisation and is unable to elicit tyrosine phosphorylation of certain substrates [Straus and Weiss (1992)]. Despite the pre-requisite of tyrosine phosphorylation for its association with PI 3-kinase, CD28 has been demonstrated to couple to PI 3-kinase in *lck* deficient cell lines [Lu *et al.* (1994)]. Thus, it has been suggested that there are at least two PTK pathways activated in response to CD28 ligation, only one of which is dependent on $p56^{lck}$ activity. Further to the proposed involvement src family protein tyrosine kinases in CD28 signal transduction, a member of the Tec family kinase $p72^{ITK/EMT}$ has been shown to be associated with CD28, in the Jurkat T cell line following ligation by mAb, in the presence or absence of

cross linking [August *et al.* (1994)]. ITK has been reported to negatively regulate T cell proliferation induced by the CD28 mediated costimulatory signal since proliferation in response to anti-CD28 antibody and sub-optimal CD3 stimulation, was enhanced in ITK deficient mouse T cells when compared to cells derived from normal animals [Liao *et al.* (1997)].

Although the identity of the CD28-directed PTK remains to be convincingly demonstrated under *in vivo* conditions, baculovirus expression systems and *in vitro* phosphorylation of CD28 peptides reveal that lck and fyn are capable of phosphorylating ^{173}Tyr within the cytoplasmic tail of CD28 [Raab *et al.* (1995)]. Furthermore, ITK appears capable of phosphorylating all four tyrosine residues in the CD28 cytoplasmic domain [King *et al.* (1997)]. These studies are summarised in Figure 1.11. Additional to the tyrosine phosphorylation sites of CD28, several sites exist which may form potential sites for phosphorylation by protein kinase C [Hutchcroft and Bierer (1996)]. These are also indicated in Figure 1.11.

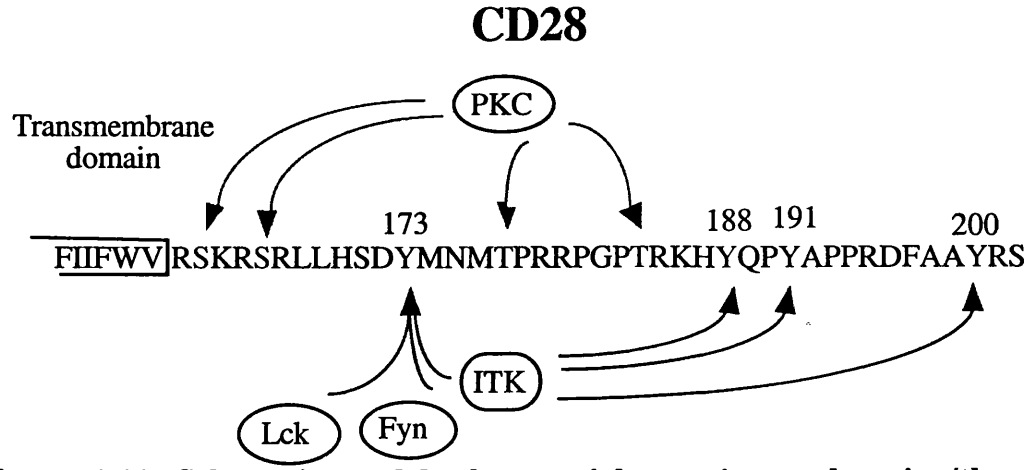


Figure 1.11 Schematic model of potential tyrosine and serine/threonine phosphorylation of CD28 cytoplasmic domain. Baculovirus expression studies in insect cells have demonstrated tyrosine 173 as a substrate of both lck and fyn, and the binding of ITK to be dependent upon lck [Raab *et al.* (1995)]. CD28 mutation studies in Jurkat cells have demonstrated ITK to phosphorylate all four tyrosine residues of the cytoplasmic tails [King *et al.* (1997)].

1.5.7.2 CD28 couples to p21^{ras}

Ras is a small, membrane bound, guanine nucleotide-binding protein that acts as a molecular switch linking receptor-associated tyrosine kinase activation to signalling events, with downstream effectors reported to include PI 3-kinase [Rodriguez-Vicinia *et al.* (1994); Hu *et al.* (1995)], Raf [Dickson *et al.* (1992)], and Ral guanine nucleotide

dissociation stimulator (Ral GDS) [Kikuchi *et al.* (1994)] (a GNEF for Ral). Ras cycles between an inactive GDP-bound state, and an active GTP-bound state. Thus Ras activity is regulated by proteins which influence the relative proportions of these bound guanine nucleotides. Guanine nucleotide exchange factors (GNEFs) activate Ras whilst GTPase-activating proteins (GAPs) down regulate Ras activity. Potentially, two routes couple CD28 to the $p21^{ras}$ pathway. Firstly, Vav is a 97 kDa GNEF identified as a substrate for CD28 induced tyrosine phosphorylation [Klasen *et al.* (1998)]. The Dbl domain of Vav confers GNEF activity and, although substrates for this activity are controversial, Vav has been demonstrated to have GNEF activity for Ras at least *in vitro* [Gulbins *et al.* (1993)]. Secondly, Grb2 is an adaptor protein which associates with a GNEF known to activate $p21^{ras}$ namely, Sos. Grb2 has been shown to associate, albeit at low affinity, with CD28 via the PI 3-kinase binding motif $(p)^{173}$YMNM [Schneider *et al.* (1995a)], which corresponds to the concensus binding motif (p)YXNX for Grb2 [Songyang *et al.* (1993)].

The CD28 mAb, CD28.2 can activate the $p21^{ras}$ pathway since it has been shown to induce an increase in ras-GTP complexes and the phosphorylation of both Vav and the Grb2/Sos-associated protein p36 [Nunes *et al.* (1994)]. The functional relevance of CD28 coupling to the $p21^{ras}$ pathway is called into question however, by the observation that binding of the natural ligand B7.1, fails to induce tyrosine phosphorylation of either Grb2 or p36 [Nunes *et al.* (1994)], although tyrosine phosphorylation of Vav can be detected after both Ab or ligand stimulation of CD28. Thus CD28 can potentially couple to the $p21^{ras}$ pathway, as demonstrated by the CD28.2 mAb, however it may not activate Ras following ligation by B7.

1.5.7.3 CD28 activates acidic sphingomyelinase.

CD28 has been reported to activate acidic sphingomyelinase (A-SMase)[Boucher *et al.* (1995)], catalysing sphingomyelin hydrolysis to phosphorylcholine and the putative signalling molecule ceramide. Pro-inflammatory cytokines such as TNF and IL-1, activate both A-SMase and N-SMase, whilst CD28 was observed to activate A-SMase in the absence of N-SMase activation, a property which distinguishes its signalling from that of pro-inflammatory cytokines. Many signalling pathways ascribed to CD28 also appear to be engaged by the TCR, however ligation of the TCR has not been demonstrated to activate A-SMase. Thus activation of A-SMase may donate an element of specificity to the CD28 derived signal. A number of putative targets for ceramide have been identified including ceramide activated protein phosphatase (CAPP) [Dobrowsky and Hannun

(1992)], ceramide activated protein kinase (CAPK) [Mathias *et al.* (1991)], JNK [Westwick *et al.* (1995)], PKCζ [Hannun and Obeid (1995)], the proto-oncogenes vav and ras [Gulbins *et al.* (1995)], and caspase 3 [Smyth *et al.* (1996)]. Such a repertoire of downstream targets enable ceramide to influence a wide range of biological processes including apoptosis, inhibition of proliferation, differentiation and protein secretion [Hannun (1994)]. Whether ceramide signals trigger positive or negative outcomes are likely to be influenced by the context and location of ceramide generation, for example, ceramide induced apoptosis in Jurkat cells is converted to a proliferative response in the presence of DAG [Hannun (1994)].

1.6.1 Structure and function CD95.

CD95, also known as Apo-1 or Fas, comprises of 325 amino acids with a signal sequence at the N terminal and a central transmembrane domain [Nagata (1994)] indicative of a type 1 membrane protein. It has a molecular weight of 45 kDa and is encoded by a sequence which maps to human chromosome 10q24.21 [Nagata (1994)]. CD95 is a member of the TNFR/NGFR superfamily, comprising over more than twelve receptors, further members of which include TNFR 1/2, NGFR, PV-T2, CD40, CD27, OX40 and 4-1BB [for review see Smith *et al.* (1994)]. They signal for broad spectrum of functional outcomes encompassing induction of thymocyte proliferation (TNFR 2) [Tartaglia *et al.* (1991)], immunoglobulin-class switching (CD40) [Kwabe *et al.* (1994)] and induction of apoptosis. CD95 triggers an apoptotic pathway when ligated [Itoh *et al.* (1991)]. The fundamental difference between this process and necrosis, or "accidental" cell death, lies in the fact that it is an active, gene directed process. Morphological and biochemical analyses of apoptotic cells indicate that apoptosis is accompanied by condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus and extensive degradation of chromosmal DNA into oligomers of about 180 bp. [Kerr *et al.* (1972)]. The apoptotic cells subsequently fragment into membrane bound apoptotic bodies which are rapidly phagocytosed and digested by macrophages or neighbouring cells. Apoptosis is believed to play an essential role in development, tissue homeostasis, defence against viral infection and clearance of damaged cells from multicellular organisms [Steller (1995)].

1.6.2 CD95L: "A ligand to kill".

Following the identification of CD95 as a receptor that could trigger apoptosis in susceptible cells, the identification and characterisation of a natural ligand became a key question. The ligand gene for CD95 (CD95L) was cloned and characterised by Suda and co-workers [Suda *et al.* (1994)], and found to encode a type II membrane protein comprising 270 amio acids with a molecular weight of 40 kDa. The expression of CD95L is relatively restricted and it is predomiantly found on activated T [Suda *et al.* (1995)] and Natural Killer (NK) cells [Arase *et al.* (1995)], although expression has been documented on neutrophils [Liles *et al.* (1996)], dendritic cells [Lu *et al.* (1997)] and macrophages [Badley *et al.* (1997)]. Chemical cross linking and gel filtration analysis have indicated that human soluble CD95L exists as a trimer [Tanaka *et al.* (1995)]. In addition to mediating apoptosis of target cells, T cell CD95L expression has also been implicated in the induction of cell suicide since T cells themselves express the CD95 receptor. A role for CD95L in cell suicide has been confirmed at the single cell level [Brunner *et al.* (1995)] and one such physiological trigger for such suicide is believed to be TCR engagement [Wesselborg *et al.* (1993)]. The first evidence that TCR engagement might be linked to apoptosis induction came from studies in immature thymocytes in which anti-CD3 treatment was associated with the rapid induction of cell death [Smith *et al.* (1989)]. Later work demonstrated that whilst resting mature T cells were relatively resistant to TCR-induced apoptosis, previously activated T cells could be triggered to undergo cell death when stimulated in this manner [Ucker *et al.* (1992); Wesselborg *et al.* (1993)]. In fact, sensitivity to this form of apoptosis was enhanced by cytokines such as IL-2 and IL-4 which promoted S-phase entry [Lenardo (1991)]. This phenomenon was termed activation induced cell death (AICD).

1.6.3 CD95 signal transduction: associated molecules.

CD95-mediated apoptosis occurs independently of both RNA and protein synthesis [Itoh *et al.* (1991)], suggesting this pathway exists in a preformed state. The cytoplasmic domain of CD95 comprises of 145 amino acids but lacks any recognised motifs for enzymatic activity [Itoh *et al.* (1991)], thus presumably the molecule signals via the recruitment of cellular enzymes. A 68 amino acid sequence shared with the TNFR, termed the "death domain", within the Fas cytoplasmic domain has been identified,which mediates association with other death domain containing proteins and is necessary for the transduction of death signals. The yeast two-hybrid system has been used to demonstrate CD95 interaction with death domain containing proteins such as FADD (Fas associated

death domain containing protein), also known as MORT 1 [Chinnaiyan *et al.* (1995)] and RIP (receptor interacting protein) [Stanger *et al.* (1995)]. The binding of FADD to the death domain appears to be dependent on the trimerisation of CD95 molecules. This is concordant with the observation that self association of the CD95 death domains is sufficient to induce a death signal [Boldin *et al.* (1995)]. Further CD95 associated signalling molecules have been identified in the CAPS (cytotoxicity dependent Apo-1 associated proteins) which associate with oligomerised, but not monomeric, CD95 [Kischkel *et al.* (1995)]. Whilst CAP 1 and CAP 2 have been identified as serine phosphorylated forms of FADD, CAPS 3 and 4 may represent novel apoptosis transducing molecules [Kischkel *et al.* (1995)]. An additional protein DAXX has been recently demonstrated to interact with the CD95 cytoplasmic domain, despite the lack of a death domain, and is thought to be involved with CD95 JNK activation [Yang *et al.* (1997)]. The recruitment of death domain containing proteins such as FADD, RIP and CAPS to the CD95 death domain results in the formation of a signalling complex that is collectively called the DISC (death-inducing singalling complex) [Kischkel *et al.* (1995)].

The carboxy terminus of the CD95 cytoplasmic tail comprises a 15 amino acid "salvation domain" which negatively regulates CD95 signalling [Itoh and Nagata (1993)] by interaction with the tyrosine phosphatase FAP 1 (Fas associated phosphatase 1) [Sato *et al.* (1995)]. Deletion of the salvation domain has been shown to enhance CD95 interaction with FADD and expression of FAP-1 encoding DNA leads to reduction in CD95 sensitivity. This suggests that one or more tyrosine kinases may play a role in CD95 killing. Moreover, it has previously been demonstrated that CD95 activity is inhibited by tyrosine kinase inhibitors, and ligation of CD95 leads to the tyrosine phosphorylation of a number of cellular substrates [Eischen *et al.* (1994)]. Indeed, the tyrosine kinase fyn has been demonstrated to physically associate with CD95, and activated T lymphocytes from fyn knock out mice are less sensitive to killing on CD95 ligation than cells from control animals.

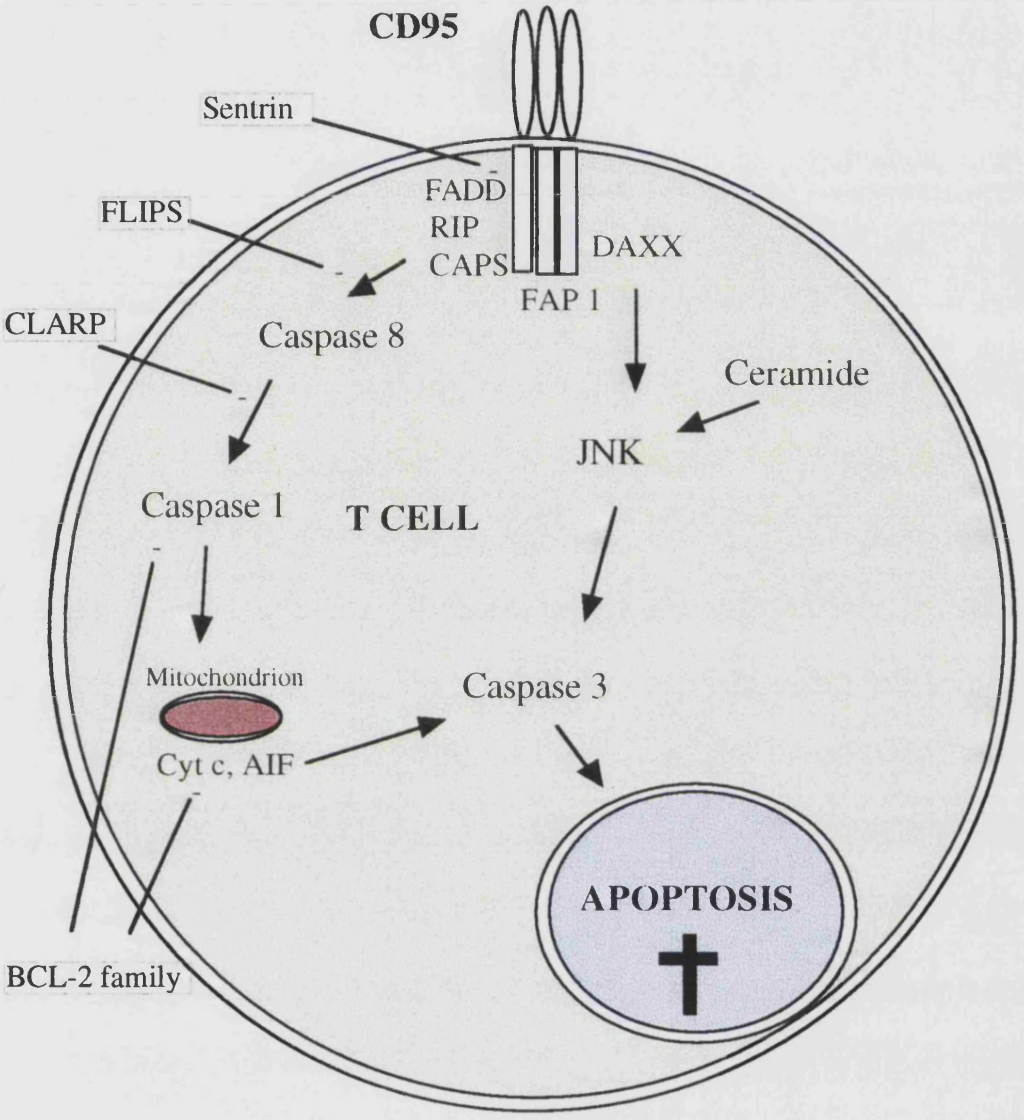


Figure 1.12 Schematic representation of CD95 mediated death signalling. The DISC (death inducing signalling complex) is formed by recruitment of death domain containing proteins (such as FADD, RIP, and CAPS) to the CD95 death domain. FAP1 is recruited to the salvation domain and may inhibit DISC formation. The DISC can recruit and activate caspase 8 (FLICE). DAXX associates with the CD95 cytoplasmic region and may be involved in JNK activation. Points of inhibition of death signalling are denoted by green border. The release of mitochondrial factors is thought to precede activation of caspase 3. Cyt c is cytochrome c, and AIF is Apoptosis Inducing Factor. Arrows do not necessarily indicate a direct link.

1.6.4 CD95 activates caspases.

A key event in the execution of apoptotic pathways is the activation of a cascade of aspartate-specific cysteine proteases (caspases). A large number of genes relevant to the cell death pathway have been identified from work on the nematode *Caenorhabditis elegans* , termed *ced* genes (*C. elegans* death genes). These include *ced*-3 (homologous with mammalian caspase 1), *ced*-9 (homologous with mammalian BCL-2) and *ced*-4, for which no mammalian homologue has been identified. CD95 may activate caspases by recruitment of caspase 8, which interacts directly with the DISC via an interaction with the death effector domains of FADD, becoming proteolytically activated as a consequence [Medema *et al.* (1997)]. Which caspases participate in apoptosis appears to be highly cell type, and stimulus, dependent. CD95 killing of T cells however, is believed to involve sequential activation of caspase 8 (FLICE), caspase 1 (ICE), and caspase 3 (PRICE), see Figure 1.12. This is supported by the observation that caspase 1-deficient mice exhibit a defect in CD95-mediated apoptosis, yet still undergo apoptosis in response to dexamethasone or γ irradiation [Cleveland and Ihle (1995)]. Furthermore, treatment with cell soluble fluoromethyl ketone caspase inhibitors such as Z-VAD-FMK (a pan-ICE nhibitor), inhibits CD95 killing in many cell types [Sarin *et al.* (1996)] implicating caspase activity as an essential step in the apoptotic pathway. Requirement of caspase activation in CD95-mediated apoptosis has also been demonstrated by the expression of *crmA* [Los *et al.* (1995); Enari *et al.* (1995)], a cytokine-response modifier gene encoded by cowpox virus that is a natural caspase inhibitor [Ray *et al.* (1992)].

A number of putative substrates for the caspases have been identified including β-actin, α-fodrin and lamin B1 [Martin *et al.* (1995b); Neamati *et al.* (1995)], the substrate responsible for committing cells to apoptosis remains unknown. The early demonstration that poly (ADP-ribose) polymerase (PARP) was a substrate for caspase 3 [Lazebnik *et al.* (1994)] suggested a possible role in the shut down of DNA repair processes. However, despite being a useful marker of apoptosis induction, PARP cleavage is dispensible for apoptosis [Wang *et al.* (1997)]. Interestingly, PKCδ represents an alternative substrate for caspase 3, and the caspase inhibitor YVAD has been reported block both PKCδ activation and DNA fragmentation in U937 cells exposed to ionizing radiation [Emoto *et al.* (1995)], suggesting a role for PKCδ in apotosis.

1.6.5 CD95 signals via ceramide.

The familial relationship of CD95 with TNFR prompted signalling comparisons which indicated some homology. One event reported to follow CD95, TNFR and CD28 ligation is the activation of certain forms of the lipid enzyme sphingomyelinase. The nature of the sphingomyelinase enzyme involved in CD95 signalling is still the subject of debate. Whilst the TNFR activates both acidic and neutral sphigomyelinase, and CD28 signalling only uses the acidic form [Boucher *et al.* (1995)], there is evidence for CD95 using both acidic [Gulbins *et al.* (1995)], and neutral sphingomyelinase [Tepper *et al.* (1995)]. Certainly, CD95 ligation is followed by sphigomyelin hydrolysis and an increase in ceramide generation [Gill *et al.* (1994)]. This appears to be necessary for cell death since cells expressing Fas, but defective in ceramide signalling, are resistant to Fas mediated apoptosis [Tepper *et al.* (1995)]. Treatment of these cells with synthetic ceramide analogues induced apoptosis, which was inhibited by treatment of cells with phorbol-12-myristate-13-acetate, suggesting opposing roles for protein kinase C and ceramide in regulation of apoptosis [Tepper *et al.* (1995)].

1.6.6 Mitochondrial changes.

A further event recently implicated in apoptotic signalling is that of mitochondrial permeability transition (PT) involving a disruption of the mitochondrial inner transmembrane potential. In viable cells, the unequal distribution of ions across the inner mitochondrial membrane generates an electrochemical potential gradient which is intimately involved in the physiological transduction of chemical energy. The disruption of this transmembrane potential has recently been identified as an early marker of cells committed to apoptosis following diverse stimuli including CD95 ligation [Castedo *et al.* (1996)] and is believed to involve a process termed permeability transition during which mitochondrial protein can leak into the cytosol. Such proteins include cytochrome c and apoptosis inducing factor (AIF) [Zamzami *et al.* (1996)]. AIF is known to proteolytically activate caspase 3 and can induce characteristic apoptotic changes when applied to isolated nuclei [Susin *et al.* (1997)]. Interestingly, inhibition of caspase 1 prevented CD95-mediated disruption in the inner mitochondrial transmembrane potential [Castedo *et al.* (1996)], demonstrating that part of the caspase cascade occurs upstream of such mitochondrial changes, whilst the activation of other proteases (such as caspase 3) may lie downstream of this process [Susin *et al.* (1997)].

1.7 Negative regulation of CD95 signalling.

Apoptotic pathways are subject to regulation by a growing number of proteins. One of the first to be identified was Bcl-2, a 26 kDa protein, associated with mitochondrial and perinuclear membranes. Although initially thought to function via an anti-oxidant method [Hockenbery *et al.* (1993)], there is now evidence that this protein may operate by altering the mitochondrial release of apoptosis-inducing factors [Kluck *et al.* (1997)]. Subsequent to Bcl-2, a whole family of related, pro- and anti- apoptotic, proteins have been identified, these proteins share a common domain of approximately 66 amino acids, comprising the Bcl-2 homology regions 1 and 2 (BH1 and BH2). Pro-apoptotic intracellular proteins include Bax (21 kDa), Bak (23 kDa), Bcl-X_S (21 kDa) and BAD [Gajewski and Thompson (1996)]. Anti- and pro-apoptotic proteins are summarised in Table 1.6.

Family member	Anti-apoptotic	Pro-apoptotic
Mcl-1	+	
NR-13	+	
A-1	+	
Bag-1	+	
Bcl-W	+	
Bcl-X_L	+	
Bcl-2	+	+
Bax	+	+
Bak	+	+
Bad		+

Table 1.6 Bcl-2 Related Proteins. Adapted from [Gajewski and Thompson (1996)]. A given family member may perform either function depending on the cell system utilised [Kiefer *et al.* (1995); Cotazzo *et al.* (1996); Middleton *et al.* (1996)]. Functional outcome may correlate with the ability to dimerise with further family members, or with how extra cellular signals regulate phosphorylation.

Proteins implicated in protection against apoptosis include BAG 1 and Bcl-X_L (29 kDa), although a given family member may perform either function, depending on the cellsystem utilised [Kiefer *et al.* (1995); Cotazzo *et al.* (1996); Middleton *et al.* (1996)]. Bcl-2 and Bcl-X_L have been suggested to function by heterodimerisation with Bax, thus preventing the formation of toxic Bax homodimers [Gajewski and Thompson (1996)]. Conversely, the dimerisation of a further protein, BAD with Bcl-2 or Bcl-X_L, seems to

liberate Bax and promote apoptosis induction [Gajewski and Thompson (1996)]. The relative role of the Bcl-2 family members in the modulation of mitochondrial events compared to the sequestration of pro-apoptotic proteins remains to be resolved.

Additional negative regulation of CD95 signalling is achieved by FLICE inhibitory proteins (FLIPS) [Irmler *et al.* (1997)]. FLIPS bind to the death affector domain (DED) of FADD, and inhibit the FADD-caspase 8 interaction [Thome *et al.* (1997)]. The FLIPS effectively act as dummy caspase 8 proteins and since they lack protease activity, prevent caspase activation inhibiting apototic signalling. Interestingly, FLIPS have been found in melanoma lesions suggesting that upregulation may occur during tumorigenesis [Irmler *et al.* (1995)]. Further negative regulation is made possible by the death effector domain-containing protein CLARP (caspase-like apoptosis regulatory protein) which interacts with and regulates caspase 8 [Inohara *et al.* (1997)] and the death domain binding protein sentrin which can inhibit both CD95 and TNFR mediated apoptosis [Okura *et al.* (1996)].

1.8 Costimulation of T cell activation by CD95.

Interestingly, there is evidence to support a positive, possibly costimulatory, role for CD95-derived signals, contrasting to its more widely accepted function in apoptosis induction. In this respect anti-CD95 antibodies have been reported to cooperate with sub optimal TCR stimulation in the induction of T cell proliferation [Alderson *et al.* (1993)], although these data await corroboration. Additionaly, T cells from CD95 defective *lpr* mice are less responsive to antigenic stimuli than are normal T cells [Davignon *et al.* (1985)], which could be interpreted as implicating CD95 in a stimulatory role. Furthermore, recent reports studying murine T cells that fail to express FADD have shown these cells to be defective in activation induced proliferation [Zhang *et al.* (1998)]. Two potentially divergent pathways must therefore be involved at the level of signal transduction in order for CD95 to be responsible for two such contrasting outcomes, proliferation or apoptosis. How this might be achieved under physiological conditions is currently unknown.

1.9 Aims and objectives.

The intracellular signalling mechanisms employed by CD28 in preventing apoptosis and driving proliferation are poorly understood, however, the profound consequence of CD28 signalling make it an attractive point for therapeutic intervention in conditions where immunomodulation would be desirable. An understanding of the biochemical nature of costimulatory signalling is of fundamental importance in the design of such therapies, and therefore requires further investigation.

At the outset of this project previous work in the laboratory had demonstrated, by direct extraction of phosphorylated lipids from radiolabelled cells, that CD28 coupled and activated PI 3-kinase [Ward *et al.* (1993)]. This correlated well with the observation that the cytoplasmic domain of CD28 contained a (p)YMNM binding motif which, in its phosphorylated state, bound the SH2 domains of p85, the regulatory subunit of PI 3-kinase [Pages *et al.* (1994); Prasad *et al.* (1994)]. There are however, a number of outstanding questions relating to the discrete biochemical events that lie proximal and distal to CD28 recruitment and activation of PI 3-kinase, thus this study was intiated with the following aims:

1) To investigate the early biochemical events regulating recruitment and activation of PI 3-kinase with particular emphasis on the role of PKC and protein tyrosine kinase lck, in light of previous studies from other groups [Raab *et al.* (1995); Hutchcroft *et al.* (1996)] that indicate these molecules exert a regulatory effect on CD28 signal transduction.

2) To investigate the effect of CD28 ligation on serine/threonine phosphorylation of CD28 and the role of PKC/ PI 3-kinase in this event, given i) the presence of a number of a number of serine/threonine residues within the CD28 cytoplasmic tail, some of which lie within potential PKC phosphorylation motifs, ii) the reported dual lipid and protein kinase activity of PI 3-kinase, and iii) that certain isoforms of PKC can be activated by PI 3-kinase.

3) To determine whether CD28-mediated activation of PI 3-kinase was sufficient to activate the known downstream effector targets of PI 3-kinase such as PKB and p70 S6 kinase.

4) To investigate the role, if any, of PI 3-kinase dependent signalling events in Fas signal transduction, given that Fas can act as a costimulatory molecule under certain conditions.

SECTION TWO

MATERIALS AND METHODS

2.1.1 MATERIALS

The materials used in this project, together with their sources, are listed below:

Reagent	Source/Comment
Adenosine triphosphate	Sigma, Poole, U.K. Stock stored at -20°C as 100 mM diluted in 100 mM Tris-HCl pH 7.4
Ampicillin	Sigma, Poole, U.K. Stock stored at -20°C at 100 mg/ml dissolved in sterile filtered ddH_2O.
Biorad protein reagent	Biorad, U.K. Used according to manufacturers instructions.
Bromophenol blue	BDH, Poole U.K.
C2 ceramide	Calbiochem Novabiochem, U.K. Stored at -20°C as 1 mM stock dissolved in DMSO.
C2 dihydroceramide	Calbiochem Novabiochem, U.K. Biologically inactive analogue. Stored at -20°C as 1 mM stock dissolved in DMSO.
Chloroform	Fisher Scientific, U.K.
Electroporation cuvettes	Northumbria Biologicals, U.K.
Enhanced Chemiluminescence reagent	Amersham International, Amersham U.K. Storage at 4°C.
Ethidium bromide	Sigma, Poole U.K. Stock 10 mg/ml dissolved in ddH_2O stored at 4°C
Ethyl formate	Fisher Scientific, U.K.
soluble Fas-Ligand	Generous gift of Dr David Sansom, University of Bath. U.K.
Flo-Scint IV scintillation fluid	Canberra Packard, U.K.
GF/A filter paper	Whatman, U.K.
Glacial acetic acid	Fisher Scientific, U.K.
Glutathione Sepharose 4B	Pharmacia U.K. Hydrated beads, stored at 4°C and washed in phosphate buffered saline (PBS) before use.

H7	Calbiochem-Novabiochem, U.K. Stock stored at -20°C as 1 mM dissolved in ddH_2O.
Heparin	Purchased as Monoparin, 5000 U/ml from C.P. Pharmaceuticals Ltd., Wrexham U.K.
Histone H2B	Boehringer Manneheim, Germany. Stock stored at -20°C at 1 mg/ml in 20 mM Tris pH 7.5.
Hydrochloric acid	BDH, Poole, U.K.
Human recombinant (hr) IL-2	Generous gift from Dr Ivan Lindley, Sandoz, Vienna, Austria.
Ionomycin	Calbiochem-Novobiochem, U.K.
IPTG (Isopropyl β -D-thiogalactopyranoside)	Sigma, Poole, U.K. 1 M stock prepared by dissolving 238.3 mg in 1 ml water, stored at -20°C.
Lymphoprep	Nycomed, Birmingham, U.K.
LY294002	Calbiochem Novabiochem, U.K. Stored at -20°C as 5 mM stock dissolved in DMSO.
Molecular weight markers	Gibco BRL, Paisley, U.K.
Methanol	Fisher Scientific, U.K.
N-butanol	BDH, Pool, U.K.
Nitrocellulose protran BA85 blotting membrane	Schleicher and Schuell, Dassel, Germany.
NP40	Fisher Scientific, U.K.
Optiphase scintillation fluid	Canberra Packard, U.K.
PD10 columns	Pharmacia U.K. Desalting columns packed with sephadex G-25.
PD98059	Kind gift of Dr Alan Saltiel, Parke-Davis, USA. Stock stored at -20°C, 5 mM dissolved in DMSO.
Petroleum ether	BDH, Poole, U.K.
Phenyl methylsulphonyl fluoride (PMSF)	Sigma, Poole, U.K. Dissolved in acetone, stored as 100 mM stock at -20°C.
4α-phorbol	Sigma, Poole, U.K. Stored as 5 mM stock in DMSO at -20°C.
Phosphate free DMEM	Sigma, Poole, U.K.
Phorbol-12-myristate-13-acetate (PMA)	Calbiochem-Novabiochem, U.K. Stored as 5 mM stock in DMSO at -20°C.
Propan-1-ol	Fisher Scientific, U.K.

Protein A sepharose CL4B	Pharmacia, U.K. Prepared according to the manufacturers instruction, stored sterile at 4°C.
Protein G beads	Sigma, Poole, U.K.
Protein Kinase Inhibitor	Sigma, Poole, U.K. Stored at -20°C as 5 μM stock diluted in 0.05% BSA.
Protogel acrylamide	Flowgen, U.K.
PVDF Polyscreen	DuPont NEN research products, Boston, USA.
Rapamycin	Generous gift of Dr Catherine Bansbach (Wyeth Ayerst, Princeton, USA). Stored as solid in dark, dissolved in ethanol when needed.
Ro31/8220	Generous gift of Dr J.S. Nixon, Roche, U.K.
Rubidium Chloride	Fluka, Germany.
Silica Gel 60, TLC plates	Whatman, U.K.
Sodium hydroxide	Fisher Scientific, U.K.
Staurosporine	Calbiochem-Novabiochem, U.K. Stock stored at -20°C as 1 mM dissolved in DMSO.
Sulphuric acid	Fisher Scientific, U.K.
Tetrabutyl ammoniumhydrogen sulphate	Fluka, Germany.
Trypan blue solution (0.4 %)	Sigma, Poole U.K. Cell culture grade.
Wortmannin	Sigma, Poole, U.K. Stored at -80°C, as 5 mM stock dissolved in DMSO.
ZVAD-FMK	Calbiochem-Novabiochem, U.K. Stock stored at -20°C as 2 mM dissolved in DMSO.

PBS comprised: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl, pH7.5 made up in ddH_2O. All other reagents were purchased from Sigma, Poole, U.K. unless otherwise stated. All sterile, cell culture grade plastics and culture media were purchased from Gibco BRL (Paisley, U.K.) unless otherwise stated.

2.1.2 Antibodies

The antibodies used in this project, together with their sources, are listed below.

Antibody	Source/Comments
9.3 anti-human CD28	Generous gift of Dr Carl June (NMRI, Bethesda, USA). Used for cell stimulations, Western blotting (diluted 1:1000) and I.P.'s coupled to protein A beads
CD28.2 anti-human CD28	Generous gift of Dr Daniel Olive (INSERM, Marseille, France). Used for cell stimulation.
anti-p85α	Generous gift of Dr Doreen Cantrell (ICRF, London, U.K.) Used for Western blotting (diluted 1:1000) and I.P.'s coupled to protein G beads.
UCHT1 anti-human CD3	Generous gift of Dr Doreen Cantrell (ICRF, London, U.K.) Used for cell stimulation, Western blotting (diluted 1:1000) and I.P.'s coupled to protein A beads.
Anti-protein kinase B α	Kind gift of Dr Brian Hemmings (FMI, Basel, Switzerland). Used for Western blotting (diluted 1:100) and I.P.'s coupled to protein A beads.
Anti-protein kinase B PH, PKBβ and PKBγ	Generous gift of Dr Dario Alessi (MRC, Dundee, U.K.). Used for Western blotting (diluted 1:1000) and I.P.'s coupled to protein A beads.
Phosphospecific anti-PKB serine 473 (p) and anti PKB	Purchased in kit form from New England Biochemicals (Beverly, MA, USA). Used according to manufacturers instructions.
Phosphospecific anti-p38 ^{182}Y(p) and anti-p38	Purchased in kit form from New England Biochemicals (Beverly, MA, USA). Used according to the manufacturers instructions.
CH11 anti-CD95	Purchased from TCS biologicals U.K. IgM molecule. Used for cell stimulations.
Anti-p70 S6 kinase	Purchased from Santa Cruz USA. Used for Western blotting according to manufacturers instructions.
12CA5 (Anti-HA)	Generous gift of Dr D. Cantrell (ICRF, London. U.K.). Used for Western blotting (diluted 1:1000) and I.P.'s coupled to protein G.

Anti-SHIP	Generous gift of Dr Mark Coggleshall (Ohio State University, USA). Ab raised in rabbits to SHIP residues 872-941. Used for Western blotting (diluted 1:1000) and I.P.'s coupled to protein A beads.
Goat anti-rabbit IgG-HRP Rabbit anti-goat IgG-HRP Rabbit anti-sheep IgG-HRP Goat anti-mouse IgG-HRP	Purchased from DAKO, Denmark. Used as secondary antibodies in chemiluminescent protein detection protocol diluted at 1:5000.
Goat anti-mouse-FITC	Purchased from Sigma, used as secondary antibody in FACS according to manufacturers instructions.
Sheep anti-mouse IgG coated magentic beads.	Purchased from Dynal, Norway. Used according to the manufacturers instructions.

2.1.3 Plasmids

The plasmids used in this study are listed below, together with their sources.

Plasmid	**Source/Comment**
PKCα	Generous gift of Dr Peter Parker (ICRF London, U.K.) Constitutively active mutant generated by substitution of a E for an A residue at position 25, expressed in pCO_2 vector.
PKCζ	Generous gift of Dr Peter Parker (ICRF London, U.K.) Constitutively active mutant produced by substitution of a E for an A residue at position 119, expressed in pMT-2 vector.
rCD2p110	Generous gift of Dr Doreen Cantrell (ICRF London, U.K.). Constitutively activated, myc tagged, p110 by linking to extracellular and transmembrane regions of rCD2. pEF-BOS vector. [Reif *et al.* (1996)].
rCD2p110R/P	Generous gift of Dr Doreen Cantrell (ICRF London, U.K.). PI 3-kinase inactivated by point mutation R1130P in kinase domain, myc tagged. pEF-BOS vector [Reif *et al.* (1996)].
HA-PKB	Generous gift of Dr B. Burgering (Utrecht, Netherlands) pEF-BOS vector.

HA-PKB kinase dead	Generous gift of Dr P. Tsischlis (Fox-Chase Cancer Centre, USA) pcmv-6 vector.
Δp85	Generous gift of Dr C.P. Downes (Dundee, U.K.) pcDNA3.1 vector.

2.1.4 Radioisotopes.

Radioisotopes used in this study are listed below, together with their sources. All assays utilising radio-isotopes were carried out in 1.5 ml screw capped micro tubes (Sartedt, Germany).

Radioisotope	**Source/Comment**
[γ-^{32}P]-ATP	(5 μCi/ml, 3000 Ci/mmol, in aqueous solution) purchased from NEN DuPont (Stevenage, UK). Stored at -20°C
[^{32}P]-orthophosphoric acid (^{32}Pi)	(5 mCi/ml, 8500-9120 Ci/mmol) purchased from NEN DuPont (Stevenage, UK). Stored at room temperature.
Phosphatidyl [2-^{3}H] inositol-4-monophosphate	(37 GBq/mmol, 1.0 Ci/mmol) purchased from Amersham International (Little Chalfont, U.K.) Stored at -20°C.
L-3-phosphatidyl [2-^{3}H] inositol 4,5-bisphosphate	(37 GBq/mmol, 1.0 Ci/mmol) was purchased from Amersham International (Little Chalfont, U.K.) Stored at -20°C.
L-3-phosphatidyl [2-^{3}H] inositol 1,3,4,5-tetrakisphosphate	(1.11TBq/mmol, 30 Ci/mmol) was purchased from NEN DuPont (Stevenage, U.K.). Stored at 4°C.
[^{3}H] thymidine	(1 mCi/ml, 2 Ci/mmol) was purchased from Amersham International (Little Chalfont, U.K.)

2.2 METHODS

2.2.1 Cell culture

All cells were grown at a constant 37°C in an humidified atmosphere of air supplemented with 5% CO_2. Cells were routinely passaged every two days. To maintain stocks, cell lines were frozen for long term storage under liquid nitrogen, as follows. Cells were pelleted (500 g, 10 min) and resuspended at 10^7 cells/ml in 20% FBS/10% DMSO and aliquoted into freezing vials. Vials were lowered into liquid nitrogen over a number of hours, using a Handi-Freeze freezing tray (Taylor-Wharton). When necessary, cells were rapidly thawed in a water bath (37°C), washed three times in fresh medium and returned to culture. Cell viability was determined by trypan blue exclusion, 100 µl of cells were removed from culture and diluted with 400 µl RPMI and 100 µl trypan blue solution. Cells were placed on a haemocytometer and examined under a light microscope, viable cells were those which excluded the trypan blue stain.

The cell lines used in this study are outlined below:

Cell line	Source/Comments
Jurkat J6	Generous gift of Dr Andres Alcover (INSERM, Paris, France). Leukaemic T cell line, cultured in RPMI 1640 supplemented with 10% FBS, streptomycin (50 µg/ml) and penicillin (50 U/ml).
Jurkat J16	Obtained from American Type Culture Collection (ATCC) (Rockville, USA). Grown in RPMI 1640 supplemented with 10% FBS, streptomycin (50 µg/ml) and penicillin (50 U/ml).
CTLL	Obtained from ATCC (Rockville, USA). Murine T cell line, cultured in RPMI-1640 supplemented with 10% FBS, hr-IL-2 (20 ng/ml) streptomycin (50 µg/ml) and penicillin (50 U/ml), in upright 25 cm^2 flasks.
DC27.1	Generous gift of Dr Daniel Olive (INSERM, Marseille, France). Murine hybridoma cell line stably transfected to express wild type and mutated human CD28 (as described in Figure 2.1). Cells were cultured in DMEM supplemented with 10% FBS, streptomycin (50 µg/ml), penicillin (50 U/ml), and 2-mercaptoethanol (50 µM).

CHO CHO-B7.1$^+$	Generous gift Dr D. Sansom (Bath University, U.K.). Chinese Hamster Ovary (CHO) cells, used as parental (control) cells or stably transfected to express CD80 [Sansom *et al.* (1993)]. Cultured in DMEM supplemented with 10% FBS, streptomycin (50 μg/ml), penicillin (50 U/ml), 25 mM HEPES, 0.375% (w/v) $NaHCO_3$, and the nucleosides adenosine, cytidine, uridine, thymidine and guanosine (all at 20 μM). These are adherent cells and when confluent, were passaged by trypsinisation. Briefly, cells were washed twice by rinsing with PBS (pH 7.3), before incubation with Trypsin-EDTA (Gibco BRL, U.K.) for five minutes at 37°C. Trypsinisation was terminated by the addition of 20 ml fresh culture medium, cells were passaged (routinely 1:5), washed twice and returned to culture.

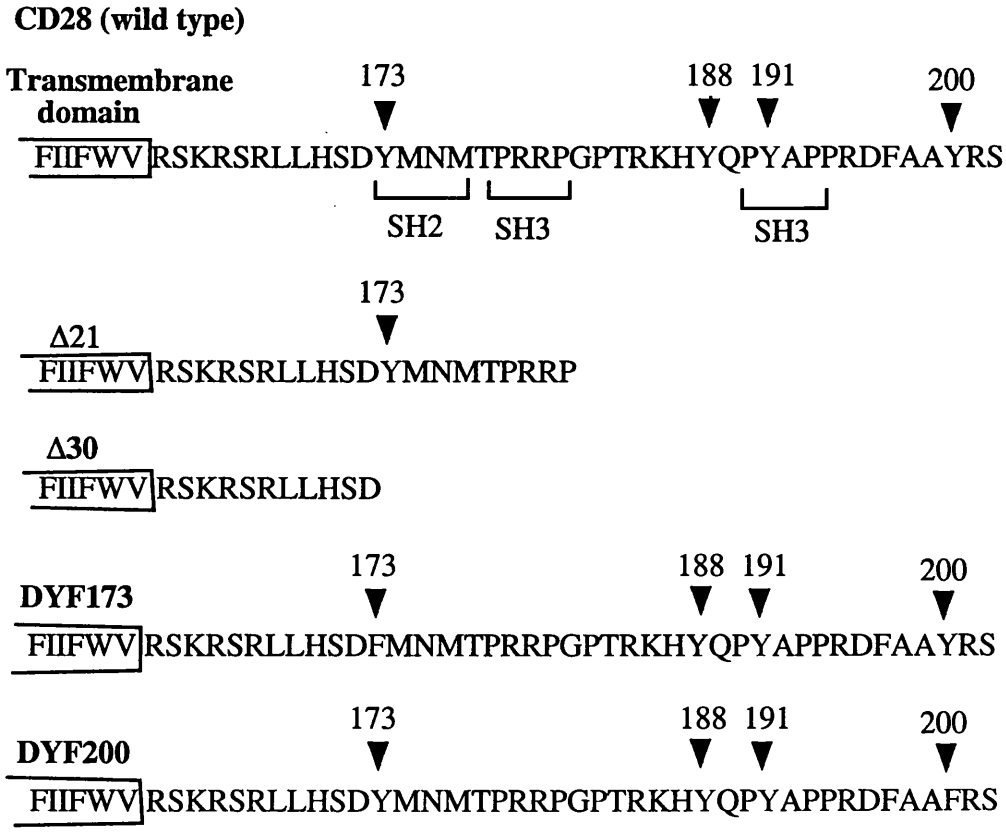


Figure 2.1 Diagrammatic representation of human CD28 mutants stably transfected into DC27.1 cell line. To examine the contribution of regions or residues of the cytoplasmic tail of CD28 to its signalling function, the above mutations were made, in human CD28, which was then expressed in DC27.1 cells. Substitution of tyrosine for phenyl alanine is structurally conservative. Transfected cell lines were found to express equivalent amounts of CD28, and routinely checked for CD28 expression, by FACS analysis.

2.2.2 Preparation of T cell blasts.

T lymphoblasts were produced essentially as described by Smith and Cantrell (1985). Samples of peripheral blood were collected aseptically via a 19-guage butterfly cannula from normal healthy donors into sterile disposable 60 ml syringes containing 50 U/ml blood heparin. After collection the blood was mixed thoroughly by gentle inversion of the syringe before dilution with an equal volume of RPMI 1640. 35 ml of the diluted blood was layered onto 15 ml of Lymphoprep in a sterile disposable 50 ml tube. The samples were centrifuged at 500 g, for 30 minutes at room temperature, and the rotor allowed to stop without braking. The mono-nuclear cell rich interface was carefully removed with a sterile pipette and washed three times by centrifugation at 500g for five minutes. The peripheral blood mononuclear cells (PBMCs) were then resuspended, in the original blood volume, in RPMI 1640 supplemented with 10% heat inactivated FBS, streptomycin (50 μg/ml), penicillin (50 U/ml) and Phytohaemaglutinin A (PHA) 1 μg/ml. After 72 hours culture at 37°C, the cells were washed three times by centrifugation at 500 g and resuspended in RPMI 1640 supplemented with 10% heat inactivated FBS, streptomycin (50 μg/ml), penicillin (50 U/ml) and hrIL-2 (20 ng/ml). Thereafter, the cells were treated with fresh medium supplemented with IL-2 every third day. Cells were starved of IL-2 for 48 hours before use. These cells, having been induced to express the IL-2 receptor by PHA treatment, and maintained with IL-2, no longer produce endogenous IL-2, but retain an ability to respond to exogenous IL-2 [Smith and Cantrell (1985)].

2.2.3 Purification of peripheral blood primary T cells.

T cells were purified by immunomagnetic depletion as described by Sansom *et al.* (1993). PBMCs were isolated from the blood of normal healthy donors as above. These were then further purified by resuspension, in the original blood volume, in RPMI 1640 supplemented with 10% FBS and incubated in sterile culture flasks at 37°C for 60 minutes. Non-adherent cells were pelleted and resuspended in 15 ml centrifuge tubes in 1 ml RPMI 1640 for each 100 ml original blood volume supplemented with the following antibodies at 1 μg/ml: monoclonal anti-CD19 (B-lymphocyte marker), anti CD14 clone UCHM-1 (monocyte and macrophage marker), and anti-MHC-II DR- clone L243 (antigen presenting cell marker). The cells were incubated with the antibodies for 60 minutes at 4°C with gentle rotation. Antibody tagged cells were then washed twice, to remove unbound antibody, and resuspended in RPMI (2 ml/100 ml original blood

volume) with 2-4x 10^6/ml magnetic beads M-450, 4.5 μm diameter, coated with sheep anti mouse IgG antibody (Dynal, Merseyside, U.K.). After 45 minutes rotation at 4°C, the cells were diluted in 10 ml RPMI 1640 and passed over a magnet three times for one minute. Unbound cells were removed with a sterile pipette and counted under a light microscope. FACS analysis confirmed that the resultant cell population were >95% T cells as judged by positive staining with UCHT1.

2.2.4 Preparation of electrocompetent cells.

All molecular biology techniques were carried out with reference to protocols contained in "Molecular cloning: A laboratory manual." (1989) Sambrook *et al.* Bacterial *Eschericia coli* DH5α cells were streaked onto a warmed Luria broth (LB) agar plate, from frozen stocks, and grown at 37°C overnight. A single colony was used to inoculate 5 ml of Luria broth, and grown in a shaking incubator at 37°C overnight. The overnight culture was subcultured 1:100 in 100 ml LB and grown until the OD550 nm reached 0.48. The cells were incubated on ice for 5 minutes before centrifugation at 6000 rpm in a Beckman JA14 rotor, for 5 minutes at 4°C. Cells were resuspended in 40 ml solution I (30 mM KCl, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol pH 5.8) and incubated on ice for 5 minutes, before pelleting by centrifugation at 6000 rpm for 5 minutes at 4°C and resuspension in 4 ml solution II 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol pH 6.5). The cells were aliquoted at 200 μl/tube, snap frozen in liquid nitrogen and stored at -80°C until use.

2.2.5 Isolation of plasmid DNA.

Electrocompetent DH5α cells were thawed and left on ice for 10 minutes. Plasmid DNA to be isolated was added to the cells at 100 ng/200 μl cells, mixed gently and incubated on ice for 30 minutes. The cells were heat shocked at 37°C for 2 minutes and returned to ice for 2 minutes. 800 μl of LB was added to the cells which were then incubated at 37°C for a further hour before plating onto an agar plate containing 100 μg/ml ampicillin. The plates were inverted and incubated overnight at 37°C. A single colony was then cultured, overnight at 37°C with shaking, in 5 ml of LB containing 100 μg/ml ampicillin. 500 μl of the preculture was added to 250 ml of LB containing 100 μg/ ml ampicillin and incubated overnight at 37°C with shaking. The bacteria were harvested by centrifugation at 5000 rpm in a Beckman JA14 rotor, for 5 minutes at 4°C and resuspended in 20 ml ice cold GTE (glucose 50 mM, Tris-HCl 25 mM, EDTA 10 mM pH 8.0). 40 ml of freshly

prepared solution II (200 mM NaOH, 1% SDS) was added the solutions mixed well and left on ice for 5 minutes. 20 ml of ice cold solution III (3 M potassium acetate, 2 M acetic acid) was added, the solutions mixed well and left on ice for 15 minutes. The mixture was then centrifuged at 8000 rpm for five minutes at 4°C and the rotor allowed to stop without braking. The supernatant was then filtered through four layers of cheesecloth into a 250 ml centrifuge bottle, 50 ml of propan-2-ol added and the bottle incubated at room temperature for 10 minutes. Nucleic acids were then recovered by centrifugation at 5000 rpm in a Beckman JA14 rotor, for 15 minutes. The supernatant was decanted and drained by inverting the open bottle. The pellet was dried by letting the bottle stand at room temperature for 30 minutes. The pellet was resuspended in 3 ml: 10 mM Tris-HCl, 1 mM EDTA pH 8.0 (TE pH 8.0) and the plasmid DNA purified by equilibrium centrifugation in a caesium chloride-ethidium bromide gradient.

2.2.6 Purification of plasmid DNA.

Caesium chloride was added to the crude preparations of plasmid DNA, obtained as above, at 1 g/ml and mixed gently at 30°C. 80 µl from a stock solution of 10 mg/ml ethidium bromide in water was added to each ml of the CsCl/DNA mixture. This solution was centrifuged at 8000 rpm for 5 minutes at room temperature. The clear red solution produced from this spin was removed to a Quick-Seal tube (Beckman) using a pasteur pipette. The tube was filled with paraffin oil and heat sealed. The tubes were then centrifuged in a Beckman Ti 70.1 rotor at 60000 rpm for at least 24 hours at 20°C. Two bands were visible in the middle of the gradient following centrifugation, the lower, containing closed circular plasmid DNA, was collected using an 18G hypodermic needle. An equal volume of water-saturated isobutanol was then mixed with the DNA solution, and the phases mixed by vortexing. The mixture was then centrifuged in a benchtop microfuge at 1500 rpm for 3 minutes at room temperature, and the lower aqueous phase removed to a fresh tube. This organic extraction of ethidium bromide was repeated five times to remove ethidium bromide. CsCl was then removed from the plasmid DNA by dialysis against TE pH 8.0.

2.2.7 Transient transfection of Jurkat cells.

Cells to be transfected were removed from culture, washed three times and aliquoted at 1.5×10^7/500 µl RPMI 1640. Samples were placed in an electroporation cuvette with 10 µg of desired DNA prior to electroporation at 310V, 960 µF in a BioRad Gene Pulser.

Cell samples were pooled, resuspended in RPMI 1640 supplemented with 10% FBS and cultured over night. Plasmid expression was determined according to the plasmid. HA-tagged proteins were detected by immunoblotting with the 12CA5 Ab, and rCD2 containing chimaeras were detected by flow cytometry analysis with the Ox34 Ab.

2.2.8 Cell lysis and immunoprecipitation

Cell lysis was carried out essentially as described by Ward *et al.* (1992). Cultured cells were harvested, washed three times in RPMI 1640 and resuspended at the desired concentration. After appropriate stimulation the cells were lysed by the addition of an equal volume of 2 x ice cold lysis buffer: 137 nM NaCl, 20 mM Tris pH 7.5, 1 mM $MgCl_2$, 1 mM CaCl, 1% Nonidet-P40, supplemented with phosphatase and protease inhibitors 150 μM sodium orthovanadate, 100 μg/ml leupeptin, 100 μg/ml pepstatin A and 200 μM phenylmethylsulphonyl fluoride (PMSF). The samples were rotated at 4°C for ten minutes before centrifugation for 5 minutes at 13,000 g at 4°C and removal of the supernatant (cellular lysate) to a fresh tube. All cellular lysates and purified proteins were kept at 4°C, unless otherwise stated.

Where whole cellular lysates were required for analysis, the cellular proteins were precipitated by addition of 600 μl ice-cold acetone to 500 μl cellular lysate, incubation at 4°C for 15 minutes before centrifugation for 5 minutes at 13,000 g in a Beckman F2402 rotor at 4°C. The supernatant was aspirated and proteins dried of acetone *in vacuo*. Where immunoprecipitates were required, the protein of interest was isolated from the cellular lysate by incubation with protein A or G beads coupled to an appropriate antibody. Protein A sepharose beads were obtained from Pharmacia as a dried powder which was hydrated by the addition of 12.5 ml distilled water to 1.5g dried beads. Protein G sepharose beads were obtained from Sigma in a prehydrated form, stored in methanol. When required, 500 μl suspensions of protein A or G sepharose beads were washed three times with 1 ml PBS and then resuspended as a 50% suspension in PBS. Antibodies were coupled to protein A or G sepharose beads by incubating 50 μg of the required antibody with 500 μl of a 50% suspension of beads and 500 μl PBS. This mixture was rotated at 4°C for two hours. Unbound antibody was removed from the beads by washing three times with 1 ml PBS. Antibody coupled beads were resuspended as a 50% suspension in PBS and stored at 4°C until required. Cellular lysates were pre-cleared by rotation with 20 μl of a 50% suspension of protein A or G beads, at 4°C for one hour. The beads were then sedimented by centrifugation and the supernatants removed to fresh tubes. To immunoprecipitate a protein of interest, a 20 μl 50%

suspension of protein A or G sepharose beads, coupled with the appropriate antibody, was rotated with the pre-cleared cellular lysate at 4°C for two hours. Beads were sedimented by centrifugation and supernatant removed by aspiration.

2.2.9 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

Protein samples were resolved essentially as described by Laemmli (1970), according to protocols previously established in our laboratory. Proteins to be analysed were solubilised by boiling for 5 minutes in SDS-PAGE sample buffer consisting: 3% SDS (w/v), 10% glycerol (v/v), 200 mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol coloured as appropriate with bromophenol blue.

Where large (15 cm vertical slab) gels were required , 7-17% acrylamide gradient gels were prepared using vertical slab gel units (model SE400), Hoefer Scientific Instruments, San Francisco). These gels were run at 75V overnight. The acrylamide gels were prepared as below. Where minigels were required BioRad Mini Protean II equipment was used (BioRad laboratories, Hemel Hempstead, UK) and 10% homogeneous gels were run, prepared as detailed in the Table 2.1. These gels were electrophoresed at 75V for two hours.

Component	7% (ml)	17% (ml)	10% (ml)	Stacking gel
Protogel	3.5	8.5	10	1.6
1M Tris pH 8.8	5.6	5.6	11.25	-
Water	5.8	-	8.15	7.6
x1 Upper buffer	-	-	-	3.1
Sucrose	-	1.5g	-	-
10% SDS	0.075	0.075	0.3	-
10% APS	0.15	0.15	0.3	0.063
TEMED	0.015	0.015	0.012	0.012

Table 2.1 Preparation of solutions for casting SDS-PAGE gels. Minigels were run as 10% homogeneous acrylamide. 15 cm vertical slab gels were run as 7-17% acrylamide gradient gels prepared with a 30 ml SG series gradient mixing chamber (Hoeffer Scientific Instruments).

Upper buffer was prepared as a x4 stock consisting: 0.5 M Tris, 0.4% SDS pH 6.8. APS and TEMED were added immediately prior to casting the gels. Once cast, the gels were overlayered with water-saturated isobutanol and left to polymerise. The isobutanol was

then washed off with water and stacking gels poured with appropriate combs in place. For both gel systems stacking gels were prepared as above and running buffer consisted 192 mM glycine, 25 mM Tris and 0.01% SDS.

Gels to be fixed were incubated in fixing solution (propan-2-ol:water:glacial acetic acid, 25%:65%:10%, v/v/v) for two hours. The gels were then placed on Whatman 3MM filter paper and dried on a BioRad gel dryer (model 583) for two hours. Radiolabelled proteins were visualised by autoradiography. Gels to be stained with Coomassie blue were immersed in Coomasie blue stain (0.1% (w/v) Coomassie brilliant blue R, dissolved in 40% methanol/7% acetic acid/53% distilled water) for four hours with gentle agitation. Destaining was achieved by decanting the Coomassie stain and immersing the gel in copious changes of destaining solution (20% ethanol/7.5% acetic acid/72.5% distilled water v/v/v).

2.2.10 Detection of proteins by Western blotting.

15 cm gels were transferred to "Polyscreen" PVDF membrane (pre-wetted according to the manufacturers instructions) in a BioRad Transblot cell at 200 mA overnight in transfer buffer consisting 192 mM glycine and 25 mM Tris. Mini gels were transferred to nitrocellulose membranes under semi-dry conditions in transfer buffer consisting 39 mM glycine, 48 mM Tris base, 0.0375% SDS and 20% methanol. The proteins were transferred at 0.8 mA/cm^2 of gel for 90 minutes. Non-specific protein interactions were blocked by incubating the membranes in 5% Marvel milk powder in PBS for two hours at room temperature. Membranes were washed by rinsing at least three time for ten minutes in 0.05% Tween 20 (v/v) diluted in PBS (PBS-T). All antibodies were diluted into a sterile filtered solution of 0.05% marvel in PBS with 0.1% NaN_3. The membranes were incubated with primary antibody for at least two hours with gentle agitation, followed by recovery of the primary antibody and rinsing, as before, prior to incubation with the secondary antibody for at least 45 minutes. The membranes were then washed with copious changes of PBS-T before visualisation with Amersham ECL western blotting reagents, according to the manufacturers instructions.

Western blots to be reprobed with a second antibody were submerged in membrane stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 60°C for one hour with occasional agitation. The blots were washed with copious changes of 200 ml PBS-Tween at room temperature, before reprobing as above.

2.2.11 Metabolic labelling of cells with [^{32}P] Pi.

Cells were labelled with [^{32}P] Pi as previously described [Ward *et al.* (1993)]. Cells to be labelled were removed from culture and depleted of intracellular phosphate by 3x 10 min incubations in phosphate free Dulbeccos Modified Eagle Medium supplemented with 20 mM HEPES, pH 7.2 at 37°C. The cells were then incubated in 10ml phosphate free DMEM supplemented with 5% FBS (previously dialysed against saline), 50 μM HEPES and 100 μCi/ml ^{32}P-labelled orthophosphoric acid, at 37°C for 3 to 4 hours.

2.2.12 Phosphoamino acid analysis of proteins

Proteins of interest were immunoprecipitated from cells radiolabelled with [^{32}P] Pi as above (section 2.2.11) separated by SDS-PAGE and using 15 cm gels and transferred to PVDF membrane as previously described (section 2.2.6). The blot was then autoradiographed to determine the position of the labelled bands, which were then excised, soaked in methanol and washed in distilled water. Bound protein was hydrolysed in 6M HCl (150-250 μl) for one hour at 110°C. The hydrolysate was dried in a speedvac (Savant), and redissolved in 5 ml of 0.1 M acetic acid containing 3 mg each of unlabelled phosphoserine, phosphothreonine and phosphotyrosine as standards. The phosphoamino acids were separated at 20 mA for 80 minutes on a cellulose TLC (Polygram Cell 300, Macherey-Nagel, Duren, Germany) in acetic acid/pyridine/water (10/1/189, v/v/v). The standard phosphoamino acids were detected by spraying with ninhydrin followed by baking at 110°C for 30 minutes. The labelled phosphoamino acids were visualised by autoradiography at -80°C.

2.2.13 Extraction of [^{32}P]-labelled phosphoinositide lipids.

[^{32}P]-labelled phosphoinositide lipids were extracted as previously described [Jackson *et al.* (1992); Ward *et al.* (1993)]. Cells metabolically radiolabelled (as described in section 2.2.11) were washed three times in phosphate free DMEM, resuspended in RPMI 1640 and aliquoted into 1.5 ml screw capped tubes at $2x10^7$/120μl. The cells were stimulated as appropriate at 37°C and the reactions quenched by the addition of 0.5 ml ice cold chloroform/methanol/water (32.65%/65.3%/2.15%, v/v/v) vortexing and incubation on ice for ten minutes. Phases were separated by the addition of 100 μl chloroform containing Folch lipids (20 μg/100 ml), and 100 μl 5 mM tetrabutylammonium sulphate,

2.4 M HCl. The samples were vortexed and centrifuged at 14000g for five minutes. The lower chloroform layer was carefully removed to a fresh tube containing 400 µl of 25 mM EDTA, 100 mM HCl mixture before further vortexing and centrifugation. The lower phases were removed to fresh tubes and dried *in vacuo*. The samples were then deacylated by the addition of 1 ml 25% w/v methylamine in water/methanol/butanol (44.4%/44.4%/11.1%, v/v/v). Samples were vortexed, incubated for 40 minutes at 53°C and cooled on ice for five minutes before drying *in vacuo*. This deacylation procedure renders the glycerophosphorylinositol (GroPtdIns) derivatives of PtdIns(3)*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (namely GroPtdIns(3)*P*, GroPtdIns(3,4)P_2 and GroPtdIns(3,4,5)P_3 respectively) water soluble. The deacylated lipids were then resuspended in 0.5 ml sterile water and washed by the addition of 0.6 ml n-butanol/40-60% petroleum ether/ethyl acetate (80%/16%/4% v/v). After vortexing and centrifugation (14000g for 30 seconds) the upper phase was discarded and the lower phase dried *in vacuo*.

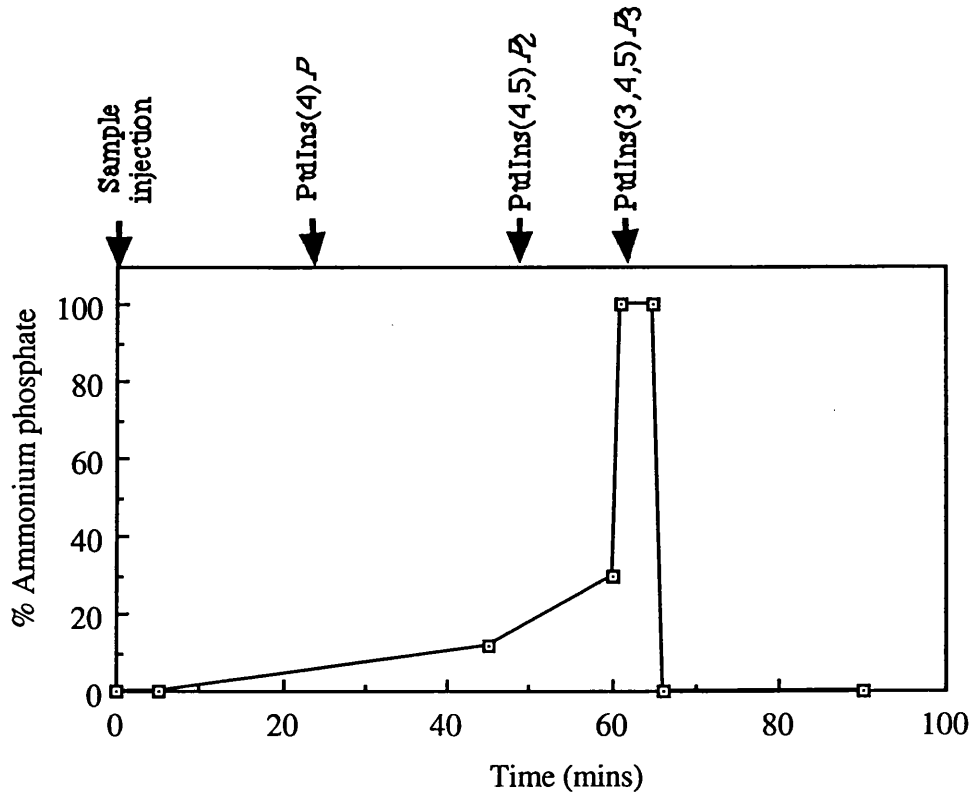


Figure 2.2 Elution programme for separation of phosphoinositides by anion exchange chromatography. Samples were applied to a Partisphere SAX column (Whatman) at a constant flow rate of 1 ml/min. Initial mobile phase (ddH2O) was progressively replaced by 1.25 M (NH4)2HPO4 (pH 3.8) as represented above. Retention times for PtdIns(4)*P* and PtdIns(3,4,5)P_3 are represented.

2.2.14 High performance liquid chromatography (HPLC).

Deacylated lipid samples, prepared as above (section 2.2.13), were resuspended in 120 µl sterile water in a sonicating water bath before being applied to an anion exchange Partisphere-SAX column (Whatman) at a flow rate of 1 ml/min. Lipids were eluted from the column using the following gradient programme represented in Figure 2.2.

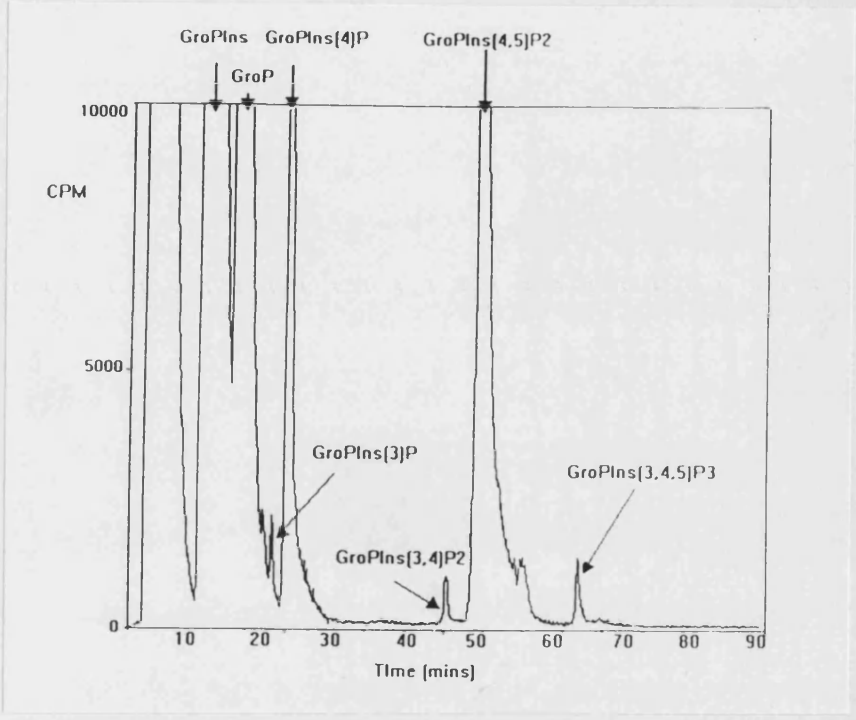


Figure 2.3 Representative trace showing the retention times of the phosphoinositides isolated from Jurkat cells. The retention times of the lipid products of PI 3-kinase were compared to previously published data [Stephens *et al.* (1991)]. Retention times can vary slightly depending on room temperature and age of column.

The anion exchange column eluate was fed into a Canberra Packard A-500 Flo-One on-line beta-radio detector where it was mixed with three volumes of Flo-Scint IV scintillation cocktail (Packard) and the results recorded and analysed using Flo-One data software (Radiomatic, USA). An example trace of the elution of lipids isolated from Jurkat cells is given in Figure 2.3. The identity of the various peaks has been previously defined [Stephens *et al.* (1991)].

2.2.15 *In vitro* lipid kinase assay

This assay was used to detect the lipid kinase activity that is present in either anti-p85α immunoprecipitates or that is co-associated with other proteins such as CD28. Cells were aliquoted at $2x10^7$/point and lysed and immunoprecipitates prepared as described in section 2.2.4. Immunoprecipitates were washed sequentially, once in NP40 lysis buffer, once in PBS, twice in 0.5 M lithium chloride, 100 mM Tris pH 7.6, once in water and finally into lipid kinase buffer (5 mM $MgCl_2$, 0.25 mM EDTA, 20 mM HEPES pH7.4). Lipid kinase activity was determined by the modified method of Whitman *et al.* (1988). The washed immunoprecipitates were resuspended in 30 µl of kinase buffer before addition of 50 µl of a mixture of PtdIns 0.1 mg/ml and phosphatidyl serine 0.1 mg/ml, dispersed by sonication in 1 mM EDTA, 25 mM HEPES pH 7.4. The reactions were initiated by the addition of 20 µCi of [γ^{32}P] ATP and 100 mM ATP, this reaction is represented schematically in Figure 2.4. After 15 minutes at 30°C the reactions were terminated by the addition of 100 µl 1M HCl and 200 µl chloroform:methanol (1:1). After vortexing and centrifugation to separate the phases, the lower organic layer was removed to a fresh tube and dried in a speedvac. The extracted phospholipids were then resuspended in 50 µl chloroform and separated by thin layer chromatography (TLC) see section 2.2.16.

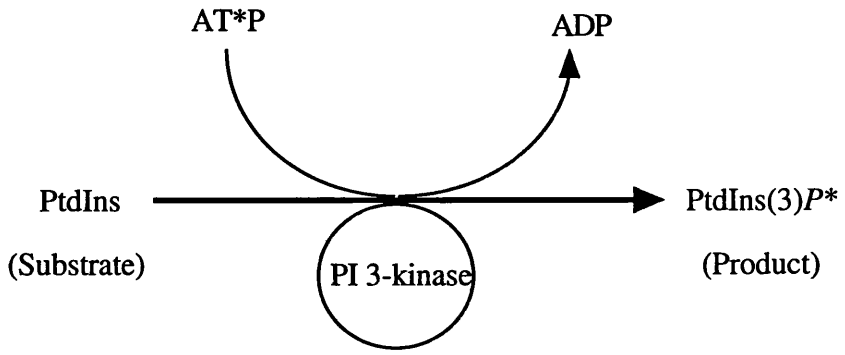


Figure 2.4 Schematic diagram of *in vitro* lipid kinase reaction.

2.2.16 Thin Layer Chromatography

Laned silica gel 60 plates (Whatman) were pre-treated by spraying with 1% sodium oxalate in water and allowed to dry. The extracted phospholipid samples, that had been re-dissolved in chloroform (section 2.2.15), were loaded onto the plates in 10 µl aliquots and placed in a pre-equilibrated solvent tank containing propan-1-ol :acetic acid (2N) (65%:35% v/v). The samples were resolved overnight and visualised by exposure to

iodine vapour prior to autoradiography. Phospholipids were identified by comparison with non-labelled standards. The phosphorylated lipid product visualised by autoradiography had been previously identified as PtdIns(3)*P* by extraction from the TLC plate and HPLC analysis by Dr Stephen Ward.

2.2.17 *In vitro* protein kinase assay

This assay was used to detect protein kinase activity associated with immunoprecipitated molecules. Immunoprecipitates were prepared as in section 2.2.8 washing three times in lysis buffer and twice in protein kinase assay buffer (100 mM NaCl, 25 mM HEPES pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 100 μM sodium orthovanadate). *In vitro* kinase assays were initiated by the addition of 20 μl of kinase buffer containing 10 μM ATP and 10 μCi [γ^{32}P] ATP. After 10 minutes at 37°C the reactions were terminated by the addition of 1 ml of kinase buffer containing 20 mM EDTA. The immunoprecipitates were then washed three times in this buffer prior to solubilisation in SDS-PAGE sample buffer. Phosphorylated proteins were separated on 7-17% acrylamide gradient gels, dried down and visualised by autoradiography.

2.2.18 Protein kinase B assay

Cells were washed, aliquoted at 10^7/point and equilibrated at 37°C prior to appropriate stimulation and lysis as above (section 2.2.8). Cell lysates were pre-cleared with protein A (or protein G, where appropriate) sepharose beads. Samples were pulsed in a benchtop microfuge and supernatants removed to a fresh tube containing either 1μg/ml anti-protein kinase B (PKB) Ab or 1 μg/ml 12CA5 Ab, before further rotation at 4°C for 60 minutes. Immunoprecipitates were produced by adding 20 μl of a 50% suspension of protein A sepharose beads to the samples and further rotation for 60 minutes. Immunoprecipitates were then washed sequentially, twice in NP40 lysis buffer, twice in LiCl 500 mM, 100 mM Tris, 1 mM EDTA pH 7.5, and finally into protein kinase B assay buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM DTT). Once in assay buffer the beads were removed to screw capped tubes, pulsed in a benchtop microfuge and supernatants aspirated. The beads were taken to dryness using a Hamilton syringe and kinase reactions initiated by addition of 15 μl kinase reaction mixture comprising as follows: 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris pH 7.5, 0.5 μM protein kinase inhibitor, 150 μg/ml H_2B, 50 μM ATP, 200 μCi/ml [^{32}P] γ-ATP. After 30 minutes at room temperature the reactions were quenched by the addition of 30 μl SDS-PAGE sample buffer and boiling for five minutes.

Proteins were then subjected to SDS-PAGE overnight as above (section 2.2.9), using 15 cm gel apparatus. Following electrophoresis the gel was cut horizontally at 40 kDa. The upper half was western blotted as above and immunoblotted with anti-PKB antibody (section 2.2.10) to demonstrate equal loading of protein. The lower half containing phosphorylated H_2B was stained with coomassie blue, destained, dried (section 2.2.9) and phosphoproteins visualised by autoradiography at -80°C.

2.2.19 Determination of JNK activity.

JNK activity was determined essentially as described by Hibi *et al.* (1993). JNK binds c-jun between residues 33-79 [Hibi *et al.* (1993)], thus a glutathione sepharose transferase (GST)-c-jun (1-135) fusion protein was used to immunoprecipitate JNK from Jurkat cells. JNK activity was subsequently assessed by the ability of JNK to phosphorylate the c-jun fusion protein, with which it was immunoprecipitated. GST-c-jun fusion proteins were prepared as follows. *Eschericia coli* cells, transfected with plasmid DNA for recombinant GST-c-jun, were grown from frozen stock overnight in 25 ml Luria broth supplemented with 50 μg/ml ampicillin (LB-amp), at 37°C in a shaking incubator. 12 ml overnight culture was inoculated into 400 ml LB-amp and grown in a shaking incubator for three hours. Expression of the recombinant protein was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The cultures were grown for a further 4 hours at 37°C in a shaking incubator. The bacterial cultures were centrifuged at 4000 rpm in a Beckman JA14 rotor, for 20 minutes at 4°C. The bacterial pellet was resuspended in 10 ml lysis buffer consisiting: PBS supplemented with 1% Triton X-100, EDTA 2 mM, PMSF 1 mM, 100 μg/ml leupeptin and 100 μg/ml pepstatin A. The pellet was resuspended completely by mixing the lysate with a pipette. The lysate was transferred to a 50 ml tube and sonicated 4x 40 seconds, with 1 minute intervals on ice. The lysate was then centrifuged at 10 000 rpm in a Beckman Ti 70.1 rotor for 10 minutes at 4°C. The supernatant was removed to a 50 ml tube and 1 ml of 50% suspension of glutathione sepharose beads in PBS was added, before the tube was rotated on a blood tube rotator for 2 hours at 4°C. The tube was centrifuged for 15 seconds at 1200 rpm and the glutathione beads washed in lysis buffer by rotation for a further five minutes. The beads were subsequently washed once in lysis bufer and three times in PBS supplemented with 2 mM EDTA. After the final wash, the glutathione beads were transferred to the top of a PD-10 column (Pharmacia). The fusion protein was eluted from the beads by competition with reduced glutathione (25 mM glutathione, 50 mM Tris-HCl pH 8.0). The glutathione solution cannot be stored, thus was prepared fresh as

required. The GST-c-jun fusion protein was eluted with 7-10 ml glutathione solution, collecting 1 ml fraction from the column. Elution fractions containing protein were pooled and dialysed against dialysis buffer (HEPES 20 mM pH 8.0, EDTA 0.2 mM, KCl 0.1 M, DTT 0.5 mM, PMSF 0.5 mM and glycerol 20% v/v) to remove the glutathione. The dialysed fusion proteins were recoupled to glutathione beads, at 1 mg protein/ 100 µl 50% suspension of beads, by rotation with the beads for two hours. The coupled beads were washed by centrifugation for 15 seconds at 1200 rpm and removal of the supernatant as before, three times in PBS and resuspended as a 50% suspension in storage buffer (HEPES 50 mM pH 7.0, NaCl 50 mM, Glycerol 50% v/v) for aliquoting at 5 µl/tube and storage at -20°C.

Cells to be used in the JNK assay were removed from culture and washed three times with RPMI 1640. Cells were aliquoted at 5x 10^6/point and stimulated as required. Cells were pulsed in a benchtop microfuge, the supernatant removed by aspiration and 200 µl whole cell extract buffer added (HEPES 25 mM pH 7.7, NaCl 0.3 M, $MgCl_2$ 1.5 mM, EDTA 0.2 mM and Triton X-100 0.1% v/v supplemented with phosphatase and protease inhibitors 150 µM sodium orthovanadate, leupeptin 100 µg/ml, pepstatin A 100 µg/ml and PMSF 200 µM). Cells were rotated at 4°C for 30 minutes before centrifugation at 13 000 rpm for ten minutes at 4°C. The supernatants were collected and 600 µl dilution buffer added (HEPES 20 mM pH7.7, EDTA 0.1 mM, $MgCl_2$ 2.5 mM, Triton X-100 0.05% v/v supplemented with phosphatase and protease inhibitors sodium orthovanadate 150 µM, leupeptin 100 µg/ml, pepstatin A 100 µg/ml and PMSF 200 µM). The supernatants were incubated on ice for ten minutes and centrifuged at 13000 rpm for ten minutes to clean the lysates. The supernatants were then removed to 1.5 ml microtubes containing 5 µl GST-c-jun glutathione beads, prepared as above. The beads were rotated for 4 hours at 4°C. The beads were then washed four times with washing buffer (HEPES 20 mM pH7.7, NaCl 50 mM, EDTA 0.1 mM, $MgCl_2$ 25 mM, Triton x-100 0.05% v/v). Kinase reactions were intiated by the addition of 30 µl kinase buffer (HEPES 20 mM pH 7.6, DTT 2 mM, β-glycerophosphate 20 mM, $MgCl_2$ 20 mM, sodium orthovanadate 0.1 mM, ATP 20 µM, 3 µCi [^{32}P]γ-ATP/reaction). Reactions were carried out at 30°C for 20 minutes, with occasional agitation. Beads were washed with 1 ml dilution buffer and proteins solubilised by boiling in 30 µl SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and visualised by autoradiography.

2.2.20 Determination of inositol polyphosphate 5-phosphatase activity.

Murine DC27.1 cells were aliquoted at 10^7/ml, stimulated as required and lysed in 1 ml ice cold NP40 lysis buffer (section 2.2.8). SHIP was immunoprecipitated from the cellular lysates using 20 µl of a 50% suspension of anti-SHIP polyclonal antibody coupled to protein A sepharose beads (section 2.2.8). Immunoprecipitates were washed twice in lysis buffer and once with phosphatase buffer: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$. Sedimented beads were resuspended in 25 µl phosphatase buffer supplemented with 5 µl/point stock [^{3}H]-Ins(1,3,4,5)P_4. Reactions were performed at 37°C for 20 minutes and quenched by the addition of 500 µl acidified chloroform (1):methanol(2). A single phase was obtained to which was added 250 µl chloroform and 250 µl 0.01 M HCl, the phases were separated by centrifugation. The upper (aqueous) phase was dried *in vacuo* and resuspended in 100 µl dH_2O. Samples were then analysed by anion exchange HPLC and the levels of [^{3}H]-Ins(1,3,4,5)P_4 and [^{3}H]-Ins(1,3,4)P_3 were quantitated using an on-line radiodetector (Canberra Packard).

2.2.21 Proliferation assay

Purified T cells were resuspended in complete medium and aliquoted at $5x10^4$ cells in 150 µl medium/well in 96 well tissue culture plates. The cells were stimulated as appropriate in quintuplicate to minimise internal variation. The TCR/CD3 complex was stimulated by UCHT1 mAb cross linked with Mouse IgG Fab fragments. The plates were incubated at 37°C in humidified air supplemented with 5% CO_2 for 72 hours before being "pulsed" with 0.5 µCi/well [^{3}H]-thymidine and returned to incubation After 18 hours, the cells were harvested onto Whatman GF/A filters using an automated cell harvester. The incoporation of [^{3}H]- thymidine was measured by placing the dried radioactive filter into 4 ml scintillation fluid (Optiphase) and counted on a β-scintillation counter (LKB).

2.2.22 IL-2 bioassay.

Quantitation of IL-2 was carried out essentially as described by Rayter *et al.* (1992). Briefly, three days after feeding, CTLL cells were removed from culture and washed twice in RPMI 1640 by 5 min centrifugation at 500 g. Cells were resuspended at 10^5 cells/ml in RPMI 1640 containing 10% FBS. A serial dilution of standard IL-2, starting at 40 U/ml was prepared and 50 µl of supernatant samples were placed in the wells of a 96

well microtitre plate. 50 µl of the CTLL cell suspension was added to each well and the plate placed in an incubator. After 18 hrs incubation 0.5 µCi [^{3}H]-thymidine was added to each well and the plate returned to the incubator. After 4 hrs the cells were harvested and [^{3}H]-thymidine incorporation measured as above (section 2.2.19). Unknown samples of IL-2 were quantitated by comparison with a standard curve prepared from the IL-2 standard samples. A representative standard curve is given below in Figure 2.5.

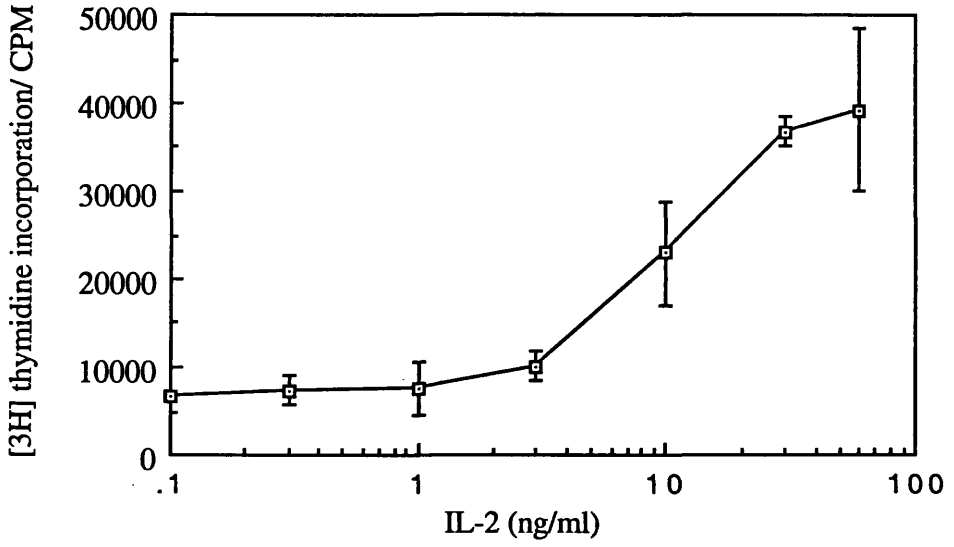


Figure 2.5 Representative standard curve for IL-2 bioassay. Data are mean and SEM of quintuplicate replicates from single representative experiment.

2.2.23 Annexin-FITC apoptosis assay.

This assay uses FITC-conjugated annexin-V to detect phosphatidyl serine, which is rapidly externalised during apoptosis [Martin et al. (1995)]. Briefly, 3x 10^5 cells were exposed to the experimental treatment for four hours at 37°C. The cells were then washed once in ice cold PBS and resuspended in 100 µl HEPES buffer (5 mM $CaCl_2$, 140 mM NaCl, 10 mM HEPES pH 7.4) containing annexin-FITC (2 µl neat/sample) and propidium iodide (final concentration 2 µg/ml). After a ten minute incubation at room temperature in the dark, samples were diluted 1:5 with HEPES buffer. Apoptotic cells were those which bound the annexin-FITC, yet excluded propidium iodide, as determined by flow cytometry analysis.

2.2.24 Flow cytometry.

Cells to be analysed by flow cytometry were removed from culture and washed three times in PBS. Cells (5x 10^5/sample) were aliquotted into FACS tubes (Beckton Dickinson) and resuspended in 50 µl primary antibody (10 µg/ml) and 25 µl FBS, antibodies used are detailed in section 2.1.2. Samples were incubated at 4°C for 30 minutes with occasional agitation. Samples were washed of unbound antibody by resuspension in 3 ml PBS and centrifugation at 500 g for 5 minutes. Samples were then incubated with 50 µl FITC-conjugated secondary antibody and 25 µl FBS, at 4°C for 30 minutes with occasional agitation. Cells were washed of unbound antibody in PBS as before, and resuspended in 200 µl PBS. Subsequent flow cytometric analysis was carried out on a FACStar Vantage (Beckton Dickinson) and data was analysed using Cell Quest software.

SECTION THREE

Early biochemical events regulating recruitment and activation of phosphatidylinositol 3-kinase by CD28

3.1 CD28 ligation results in recruitment and activation of PI 3-kinase

Signal transduction events elicited in response to CD28 ligation are relatively poorly defined in comparison to those of the TCR. CD28 has been demonstrated however, to recruit and activate the putative signalling molecule PI 3-kinase [Ward *et al.* (1993); Pages *et al.* (1994)], resulting in rapid and strong accumulation of the lipid products of PI 3-kinase which may be sufficient to activate downstream effector molecules. Activation of PI 3-kinase has been demonstrated as a pivotal event in CD28-mediated T cell proliferative reponses and IL-2 production [Ward *et al.* (1996)]. Experiments were conducted therefore, to further characterise the recruitment and activation of PI 3-kinase by CD28, before examining the activation of downstream targets.

Studies of biological functions dependent upon the activity of PI 3-kinase have been greatly advanced by the use of pharmacological inhibitors of the kinase such as wortmannin, a fungal metabolite which covalently binds to the p110 subunit [Thelen *et al.* (1995)], inhibiting both its lipid and protein kinase function. Before attempting to identify a role for PI 3-kinase in the regulation of downstream effector signalling molecules, it was important to confirm the inhibitory action of wortmannin. Accordingly, PI 3-kinase was immunoprecipitated from Jurkat cells using an anti-p85α antibody and both *in vitro* lipid and *in vitro* protein kinase function was evaluated in the presence of wortmannin. Figure 3.1.1a demonstrates that the protein kinase activity of PI 3-kinase was inhibited by wortmannin. In the absence of wortmannin (control), both the p85 and p110 subunits of PI 3-kinase are substrates for *in vitro* phosphorylation. Incubation of Jurkat cells, for ten minutes, with wortmannin at concentrations greater than 10 nM however, is observed to inhibit the phosphorylation of these proteins. An inhibitory effect on phosphorylation was also observed on addition of wortmannin to the washed immunoprecipitated proteins just prior to assay for kinase activity (Figure 3.1.1b).

Lipid kinase activity of p85 immunoprecipitates, derived from Jurkat cells, was also found to be sensitive to inhibition by wortmannin (Figure 3.1.1c). Lipid kinase activity associated with p85 immunoprecipitated from Jurkat cells, resulted in considerable phosphorylation of exogenous PtdIns substrate forming PtdIns(3)*P*. Incubation of Jurkat

cells with wortmannin (0.1 to 100 nM) was observed to inhibit PtdIns(3)*P* formation, and complete inhibition of lipid kinase activity was achieved using higher concentrations of wortmannin.

Ligation of CD28 by its natural ligand, namely B7.1, is followed by recruitment of PI 3-kinase as demonstrated in Figure 3.1.2a. CD28 immunoprecipitates from unstimulated (control) Jurkat cells do not show any co-association with p85, whilst ligation of CD28 with B7.1 however, induces rapid co-association with PI 3-kinase, as evidenced by p85 detected in CD28 immunoprecipitates from cells stimulated for one minute. This co-association with PI 3-kinase is seen to be sustained for at least 15 minutes. Recruitment of PI 3-kinase to CD28 results in an up-regulation of the enzymatic activity of PI 3-kinase, as evidenced by increased *in vitro* lipid kinase activity in CD28 immunoprecipitates. Figure 3.1.2b shows B7 treatment of Jurkat cells to induce a large increase over basal, of lipid kinase activity associated with CD28 immunoprecipitates, which is sensitive to inhibition by wortmannin (1 nM to 10 μM). Further evidence that the lipid kinase activity observed is resultant from PI 3-kinase is provided since inhibition is achieved by LY294002 (1.5 to 30 μM) [Vlahos *et al.* (1994)], another PI 3-kinase inhibitor that is structurally unrelated to wortmannin.

An alternative approach to studying the activation of PI 3-kinase is to metabolically radiolabel cells with ^{32}P inorganic phosphate, and directly extract the cellular phosphoinositide lipids in chloroform. Separation of lipids, solubilised in water by deacylation, using anion exchange HPLC methodology enables their qualitative and quantitative detection, allowing for the study of changes in concentrations of lipids induced on cellular stimulation. This may be a preferable technique since it allows for the study of kinase activity under more physiological conditions, avoiding some of the potential artifacts involved with *in vitro* kinase methodologies. Figure 3.1.2c details CD28 mediated accumulation of PtdIns(3,4,5)P_3, which is thought to be the major signalling molecule produced by PI 3-kinase activation. Lipid samples extracted from unstimulated cells reveal modest levels of PtdIns(3,4,5)P_3, indicating a basal production of the lipid in Jurkat cells. Ligation of CD28 by the natural ligand B7.1 results in a rapid accumulation of PtdIns(3,4,5)P_3. This is maximal after five minutes, and the levels of PtdIns(3,4,5)P_3 produced on receptor ligation are approximately eight-fold above basal levels. Levels of PtdIns(3,4,5)P_3 tend to decrease slightly after five minutes but remain elevated above basal levels for up to twenty minutes. Transient transfection of a dominant negative p85 (Δp85) in which the inter-SH2 domain required for interaction with the p110 catalytic subunit has been deleted, also inhibited both basal and CD28-stimulated levels of PtdIns(3,4,5)P_3 in Jurkat cells, when compared to responses obtained in cells

subjected to mock transfection conditions (Figure 3.1.2d). It is dificult however to quantify the expression of Δp85 which may lead to difficulties in interpreting the data.

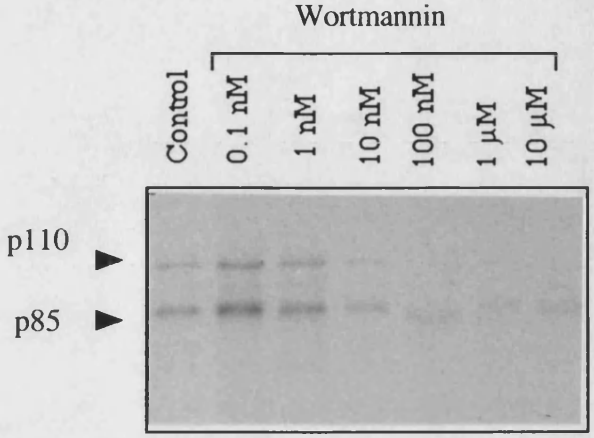


Figure 3.1.1a Protein phosphorylation of PI 3-kinase is wortmannin sensitive. Jurkat cells ($2x10^7$cells/point) were pre-treated with wortmannin at the concentrations indicated, for 10 minutes. PI 3-kinase was immunoprecipitated using an antibody to the p85 regulatory subunit. Immunoprecipitates were washed, as described, before *in vitro* protein kinase assays were performed in 20 μl kinase buffer containing 10 μM ATP and 10 μCi [γ^{32}P] ATP (see section 2.2.17). Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography.

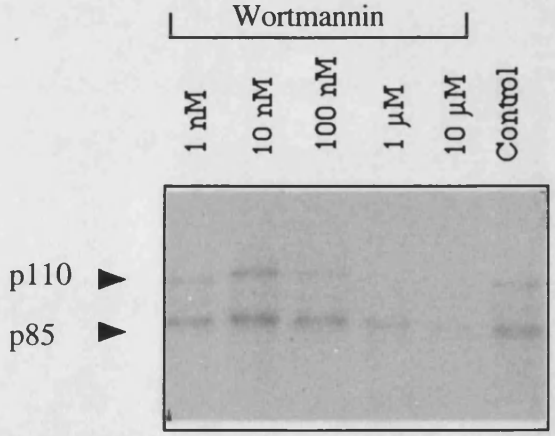


Figure 3.1.1b *In vitro* Protein kinase activity of PI 3-kinase immunoprecipitates is wortmannin sensitive. Jurkat cells ($2x10^7$/point) were lysed, and p85 immunoprecipitates were prepared and washed, as described (section 2.2.4). Immunoprecipitates were pre-treated with wortmannin at the concentrations indicated, for 10 minutes. *In vitro* protein kinase assays were performed in 20 μl kinase buffer containing 10 μM ATP and 10 μCi [γ^{32}P] ATP (see section 2.2.17). Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography.

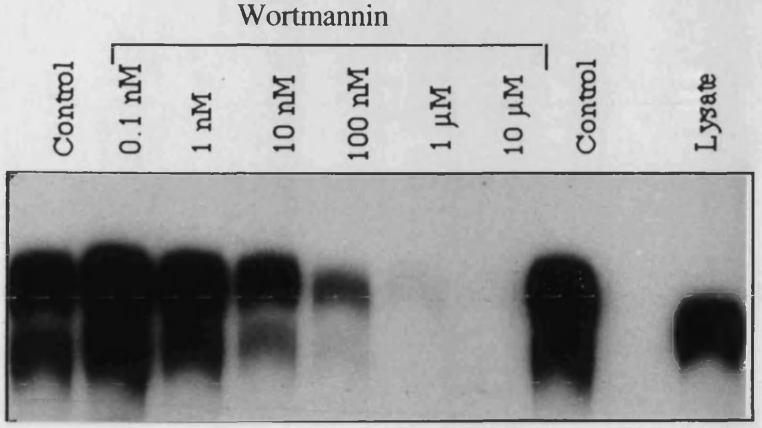


Figure 3.1.1c Lipid kinase activity associated with immunoprecipitated p85 is inhibited by wortmannin. Jurkat cells were aliquoted (10^7/point) and pretreated with wortmannin, at the concentrations indicated in the annotation, for ten minutes. Cells were then lysed in ice-cold NP40 lysis buffer and p85 immunoprecipitated with anti-p85α Ab. Lipid kinase activity was assessed by the ability of the immunoprecipitates to phosphorylate added lipid substrate (section 2.2.15), which was then separated by thin layer chromatography and visualised by autoradiography. Lysate control represents kinase activity present in 20 µl of cellular lysate. Data from single representative experiment.

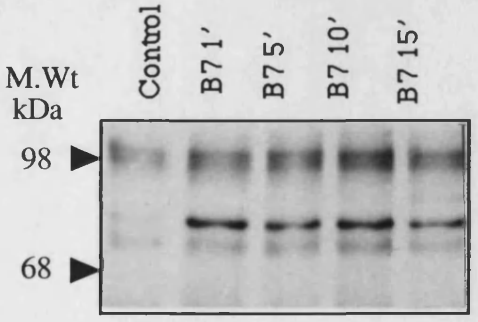


Figure 3.1.2a CD28 ligation is followed by recruitment of PI 3-kinase. Jurkat ($2x10^7$) cells were co-sedimented with CHO-B7$^+$ (10^7) cells, where indicated, for the times described in the annotation. Cells were then lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel. Resolved proteins were transferred to PVDF membrane as described (section 2.2.10) and recuitment of PI 3-kinase demonstrated by immunoblotting for the p85 subunit as described (section 2.2.10). Data is from single representative experiment.

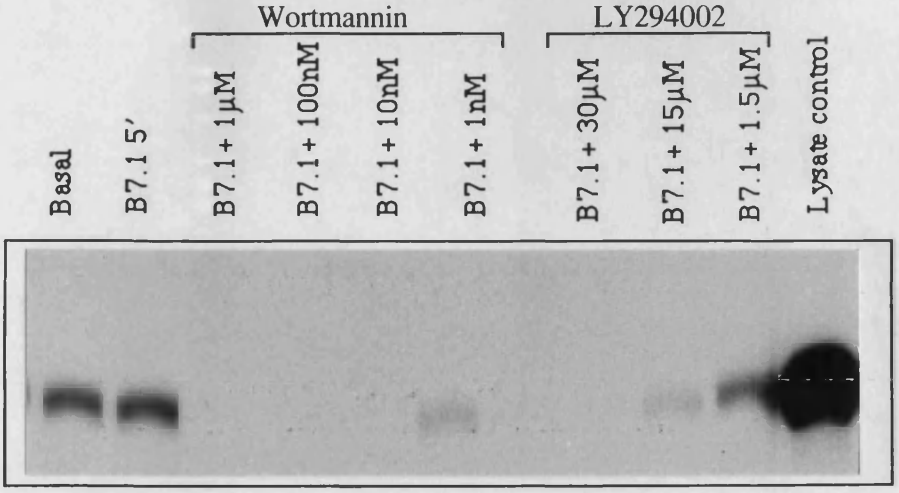


Figure 3.1.2b PI 3-kinase activity associated with CD28 is inhibited by wortmannin and LY294002. Jurkat cells ($2x10^7$/point) were co-sedimented with CHO-B7.1$^+$ cells (10^7/point) for 5 minutes before lysis in ice-cold NP40 lysis buffer. CD28 was immunoprecipitated using 9.3 anti-CD28 mAb. Immunoprecipitates were washed as described and resuspended in 30 μl kinase buffer with added PtdIns as exogenous substrate. Reactions were intiated by the addition of 100 mM ATP and 20 μCi [γ-^{32}P] ATP. After 15 minutes at 30°C, as described in section 2.2.15. Phosphorylated lipid substrate was then extracted in chloroform, resolved by TLC and visualised by autoradiography.

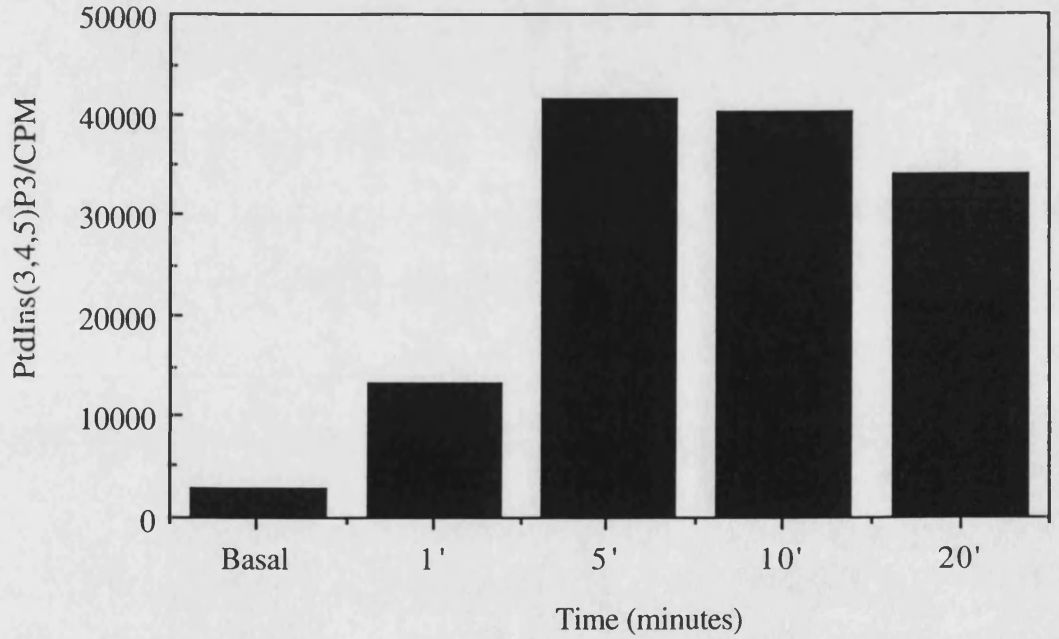


Figure 3.1.2.c Time course of CD28-mediated PtdIns(3,4,5)P_3 accumulation in Jurkat cells. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$cells/point) were co-sedimented with CHO-B7.1$^+$ cells (10^7cells/point). After incubation at 37°C, cellular lipids were extracted in chloroform, deacylated and analysed by HPLC as described (see section 2.2.14). Data expressed as absolute counts from single representative experiment

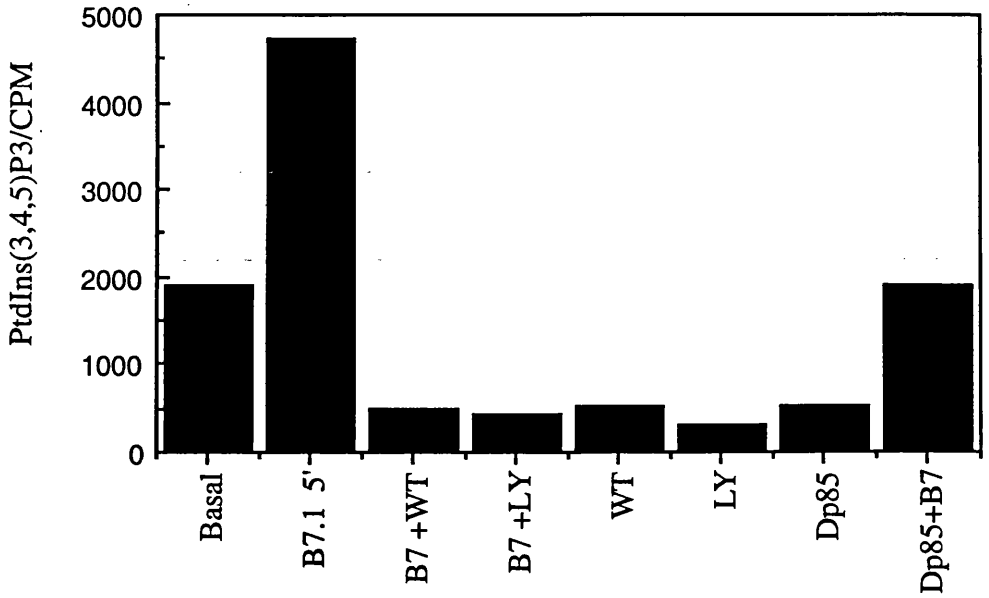


Figure 3.1.2.d CD28 mediated accumulation of PtdIns(3,4,5)P_3 in Jurkat cells is inhibited by wortmannin, LY294002 and Δp85 expression. Jurkat cells were subjected to transfection conditions or transfected with Δp85 DNA as described (see section 2.2.7) and incubated overnight.The cells were then radiolabelled and aliquoted at 5x10^{6}cells/point and incubated with wortmannin (100 nM) or LY294002 (30 μM) as above, prior to stimulation by co-sedimentation with CHO-B7.1$^+$ cells. Cellular lipids were extracted in chloroform, deacylated and analysed by HPLC as described, (see section 2.2.14). Data expressed as absolute counts from single representative experiment.

3.2 The role of Lck in PI 3-kinase recruitment and activation by CD28.

CD28 recruitment of PI 3-kinase has been demonstrated to be dependent upon the prior tyrosine phosphorylation of the YMNM binding motif, which has specificity for the SH2 domains of PI 3-kinase [Pages *et al.* (1994)]. Since analysis of the CD28 cytoplasmic domain reveals it to encode no recognised enzymatic motifs, the requirement for tyrosine phosphorylation implicates an intervening tyrosine kinase in coupling CD28 to downstream signalling cascades, and thus effectual function. Despite the influential role for this phosphorylation event in human T cells, the identity of the kinase responsible for this phosphorylation in human T cells remains to be defined. JCaM1 is a mutant of the Jurkat leukaemic T cell line, which has previously been shown to be defective in tyrosine phosphorylation and intracellular calcium mobilisation following stimulation of the T cell antigen receptor [Straus and Weiss (1992)]. This aberrant cell signalling was found to

represent a failure to express functional lck and transfection of JCaM1 cells with cDNA encoding lck restored TCR function, at least in terms of calcium signalling [Straus and Weiss (1992)]. Given that lck appears to fulfil a dual function in the signal transduction repertoire of both the TCR and CD28, experiments were performed using the JCaM1 cell line to examine the role of this tyrosine kinase in costimulatory signalling.

Firstly, it was important to confirm that the Jurkat leukaemic cell line and subclone JCaM1, expressed equivalent levels of CD28 and that JCaM1 cells failed to express lck. This was done by flow cytometry and immunoblot analyses of cells respectively. Flow cytometric analysis of the two cell lines showed them to express equivalent levels of CD28 (Figure 3.2.1a), whilst immunoblot analysis of cellular lysates derived from Jurkat and JCaM1 cells (Figure 3.2.1b), revealed Lck to be present in Jurkat cell lysates, but absent in lysates derived from identical numbers of JCaM1 cells, confirming previous observations [Straus and Weiss (1992)]. Ligation of CD28 by B7.1 in both Jurkat and JCaM1 cells, was found to induce co-association of the p85 subunit of PI 3-kinase with CD28 (Figure 3.2.1c). Following the demonstration that lck deficient JCaM1 cells display ligation-dependent recruitment of PI 3-kinase to CD28 (Figure 3.2.1c), further experiments were performed to examine whether or not the subsequent activation of the lipid kinase function of the enzyme was impaired by the absence of lck. Analysis of the [^{32}P]-labelled PtdIns(3,4,5)P_3 formed in response to CD28 ligation by B7.1, revealed an impaired lipid kinase function in JCaM1 cells (Figure 3.2.2). Co-sedimentation of Jurkat cells with CHO-B7.1$^+$ cells, was followed by an eight to ten-fold increase above basal levels of PtdIns(3,4,5)P_3. However, co-sedimentation of JCaM1 cells with CHO-B7.1$^+$ cells was followed by a four-fold increase in PtdIns(3,4,5)P_3 above basal levels (Figure 3.2.2). The discrepancy in levels of PtdIns(3,4,5)P_3 extracted from Jurkat and JCaM1 cells is unlikely reflect any general difference in cellular metabolism since the average total CPM extracted from these cells were 2.4±0.5 x10^7 (n=4) and 2.5±0.2 x10^7 (n=4), respectively. Thus, although PI 3-kinase recruitment to CD28 is not affected by the absence of lck, the subsequent activation of the lipid kinase function of the enzyme, is severely impaired.

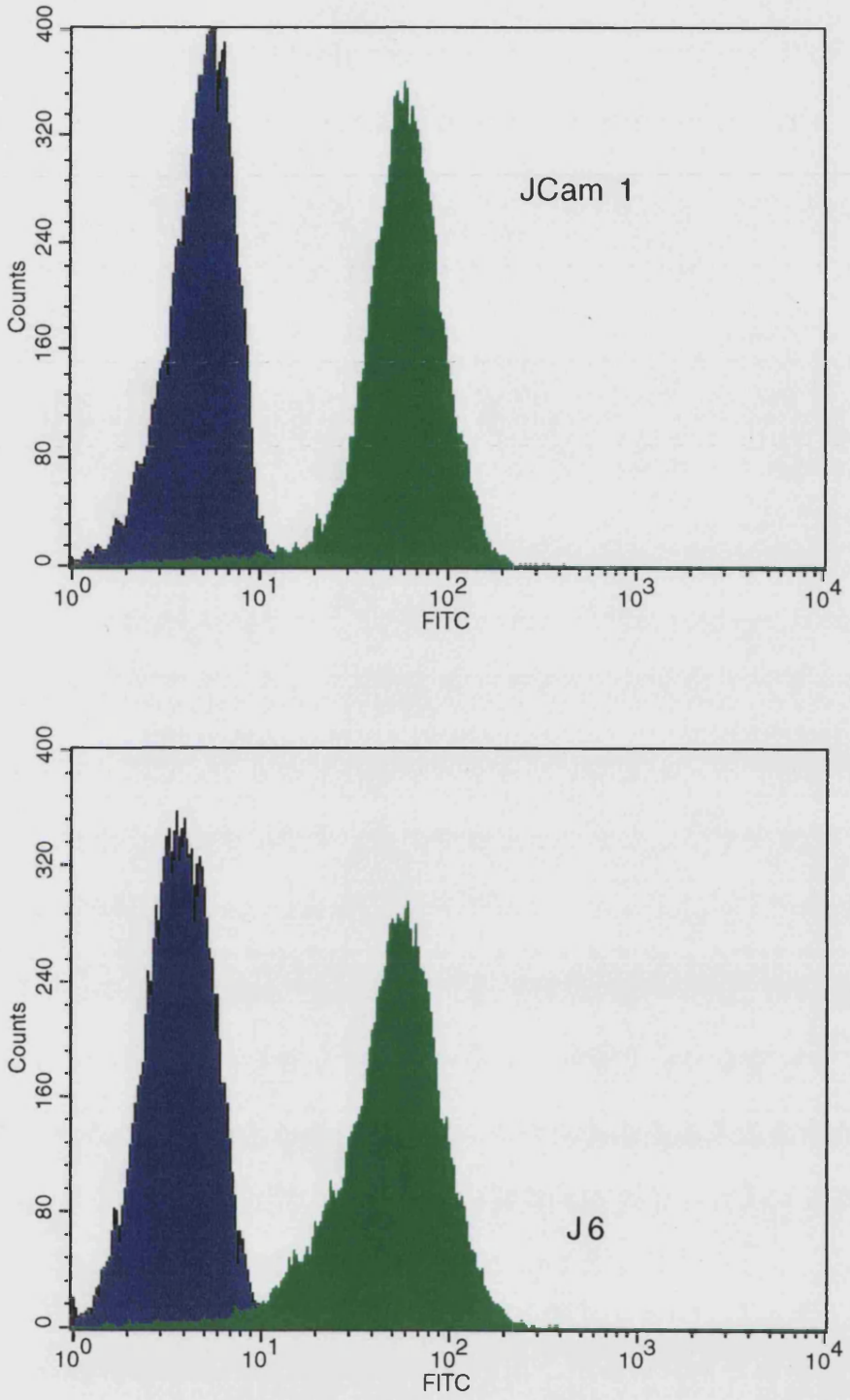


Figure 3.2.1a Jurkat and JCaM1 cells express equivalent levels of CD28. $5x10^6$ cells/point were incubated with primary antibodies, control anti-mouse IgG (10 μg/ml) (blue histograms), and anti-CD28 mAb 9.3 (10 μg/ml) (green histograms). Data from single representative experiment.

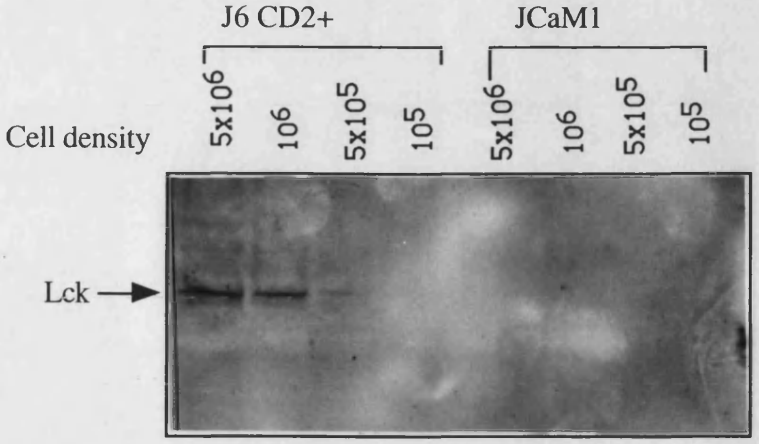


Figure 3.2.1b JCaM1 cells do not express Lck. Lysates of the Jurkat and JCaM1 cell lines were resolved by SDS-PAGE, using 10% homogeneous acrylamide gels, and transferred to nitrocellulose membranes. Immunoblotting was then performed on the Western blots using anti-lck antibody as described (section 2.2.10).

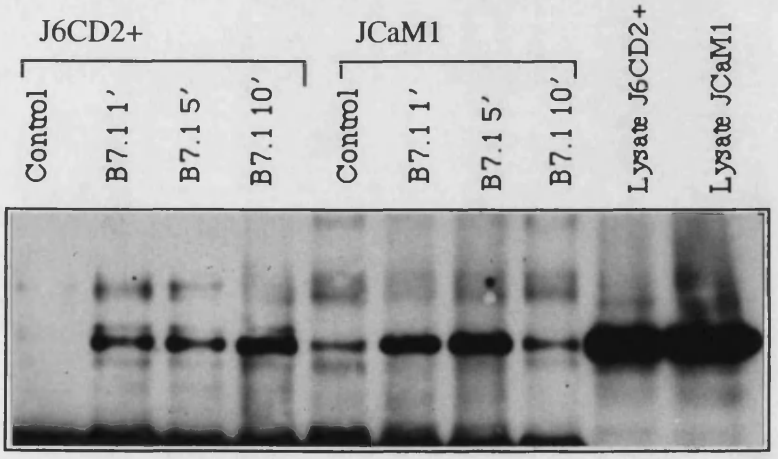


Figure 3.2.1c Failure to express Lck does not prevent ligation-dependent recruitment of PI 3-kinase to CD28. Jurkat cells ($2x10^7$/point) were co-sedimented with CHO-B7.1$^+$ cells (10^7/point), for the times indicated, before lysis with ice-cold NP40 lysis buffer. CD28 immunoprecipitates were then prepared using the 9.3 mAb. Immunoprecipitated proteins were subjected to SDS-PAGE, using 15cm, 7-17% acrylamide gradient gels. Resolved proteins were transferred to nitrocellulose membranes. Association of PI 3-kinase was assessed by immunoblotting for the presence of the p85 subunit of the enzyme. Data is from single representative experiment.

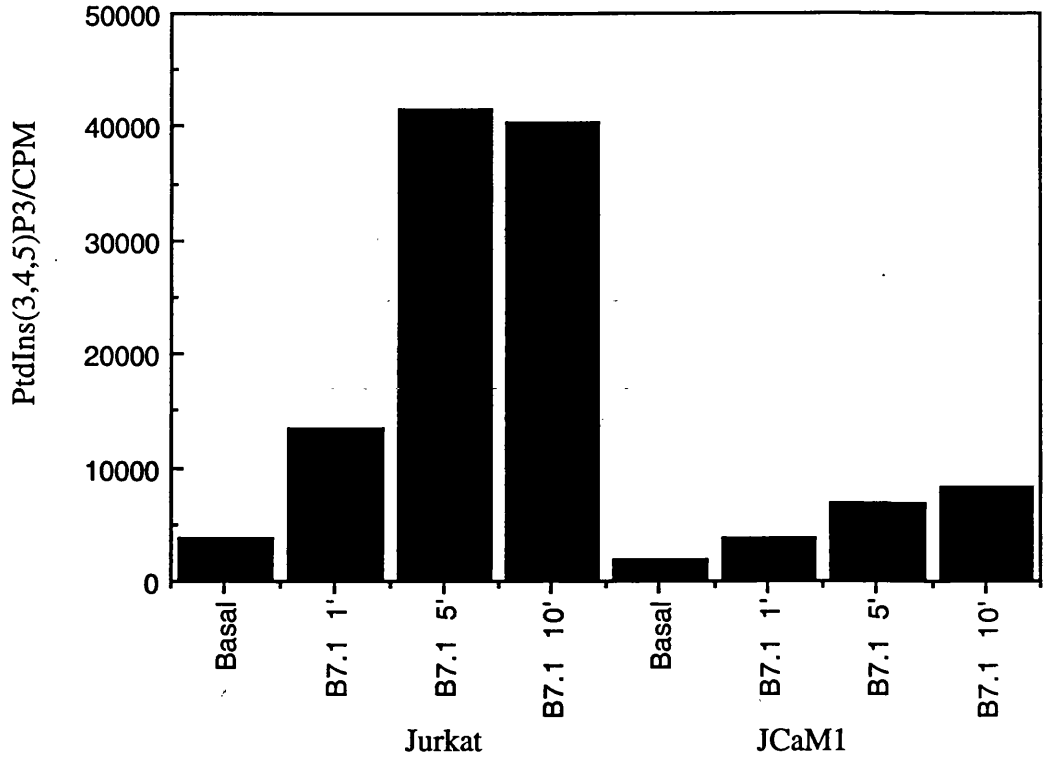


Figure 3.2.2 Failure to express Lck impairs CD28 mediated PtdIns(3,4,5)P_3 accumulation. Cells of the Jurkat and JCaM1 cell lines were radiolabelled with ^{32}P, as described (see section 2.2.11). Jurkat cells ($2x10^7$/point) were then co-sedimented with CHO-B7.1$^+$ (10^7/point) for five minutes before chloroform extraction of cellular lipids. Lipids were then solubilised in water by deacylation and subjected HPLC analysis, as described (see section 2.2.14). Data expressed as absolute counts from single representative experiment.

3.3 Effect of phorbol ester treatment on CD28 recruitment and activation of PI 3-kinase.

PMA is capable of supplying cellular signals additional to CD28 ligation, sufficient to drive IL-2 production in combination with CD28 mediated signals. It may also modulate the enzymatic activity or physical association of proteins coupled to the CD28 cytoplasmic tail. In particular, the PKC phosphorylation site around ^{177}Thr may serve to modulate the PKC-mediated regulation of PI 3-kinase binding to CD28 given its close proximity to the ^{173}YMNM binding motif.

In order to assess the effect of PKC activation on CD28:PI 3-kinase associations, immunoblotting experiments were performed on CD28 immunoprecipitates derived from resting or B7.1 stimulated cells that had been pretreated with PMA, a pharmacological activator of PKC. These immunoprecipitates were then immunoblotted using an antibody

to the p85 subunit of PI 3-kinase. These experiments revealed that the association of the p85 subunit of PI 3-kinase with CD28 following treatment with the natural ligand, B7.1 was partially inhibited by treatment of the cells with PMA (100 ng/ml) (Figure 3.3.1a). The decreased association of PI 3-kinase with CD28, in the presence of PMA, correlated with a decrease in the amount of PI 3-kinase activity associated with CD28 immunoprecipitates (Figure 3.3.1b). The inhibition by PMA of both the ligation-dependent association of CD28 with PI 3-kinase (Figure 3.3.1c) and the lipid kinase activities associated with CD28 immunoprecipitates (Figure 3.3.1d) were prevented by pretreatment of the cells with the PKC inhibitor Ro 31/8220. It should be noted however that Ro 31/8220 exerted a greater degree of protection against PMA driven down-regulation of p85 association with CD28 (Figure 3.3.1c) than it did for PMA driven down-regulation of *in vitro* lipid kinase activity associated with CD28 immunoprecipitates (Figure 3.3.1d).

Additionally, the effect of PMA treatment upon B7.1/CD28 driven PI 3-kinase activation was assessed by measuring the accumulation of PtdIns(3,4,5)P_3. The previously reported increase in PtdIns(3,4,5)P_3 after CD28 ligation can be inhibited by pretreatment of the cells with PMA (5-500 ng/ml) (Figure 3.3.2a). However the non-PKC acting phorbol ester 4-α phorbol, was without effect on CD28 mediated PtdIns(3,4,5)P_3 accumulation, a further demonstration that PMA exerts its effect via activation of PKC. Optimal inhibition was induced after 5-10 minutes of pretreatment and was sustained for at least 30 minutes (Figure 3.3.2b). Concordant with previous results (Figure 3.3.1), pretreatment of the Jurkat cells with the PKC inhibitor Ro31/8220 (5 μM) prior to addition of PMA reduced the inhibitory effects of PMA. Neither PMA or Ro 31/8220 had any detectable effect on basal levels of PtdIns(3,4,5)P_3 (Figure 3.3.2c).

PMA and CD28 are known to provide stimuli sufficient to drive T cell proliferation . Given the observation that PMA disrupts the coupling of CD28 to PI 3-kinase, it was important to establish whether PI 3-kinase activation is required for CD28 dependent activation of normal T cells in the presence of PMA. The effect of PI 3-kinase inhibitors has therefore been investigated on [^{3}H]-thymidine incorporation by normal T cells driven by PMA and CD28. In this study, concentrations of PMA greater than 0.05 ng/ml were sufficient to support [^{3}H]-thymidine incorporation in the presence of CHO-B7.1$^+$ (Figure 3.3.3a). Moreover, [^{3}H]-thymidine incorporation driven by PMA and CHO-B7.1$^+$ is optimal in the presence of 0.5 ng/ml PMA (Figure 3.3.3a), whilst sub-optimal responses are induced in the presence of higher concentrations of PMA (5-50 ng/ml). The PI 3-kinase inhibitors wortmannin and LY294002 induced partial inhibition (<60% and <50% inhibition respectively) of [^{3}H]-thymidine incorporation induced by CHO-B7.1$^+$

in the presence of 0.5 ng/ml PMA (Figure 3.3.3b and Figure 3.3.3c). Interestingly the sub-optimal [^{3}H]-thymidine incorporation observed in response to CHO-B7.1$^+$ and 5 ng/ml PMA is only weakly inhibited (less than 20% inhibition) by either wortmannin (Figure 3.3.3b and Figure 3.3.3c), whilst [^{3}H]-thymidine incorporation induced by 50 ng/ml PMA in combination with CHO-B7.1$^+$ was completely resistant to wortmannin and LY294002. In contrast, wortmannin or LY294002 completely inhibited [^{3}H]-thymidine incorporation induced by the combination of anti CD3 mAb UCHT1 and CHO-B7.1$^+$.

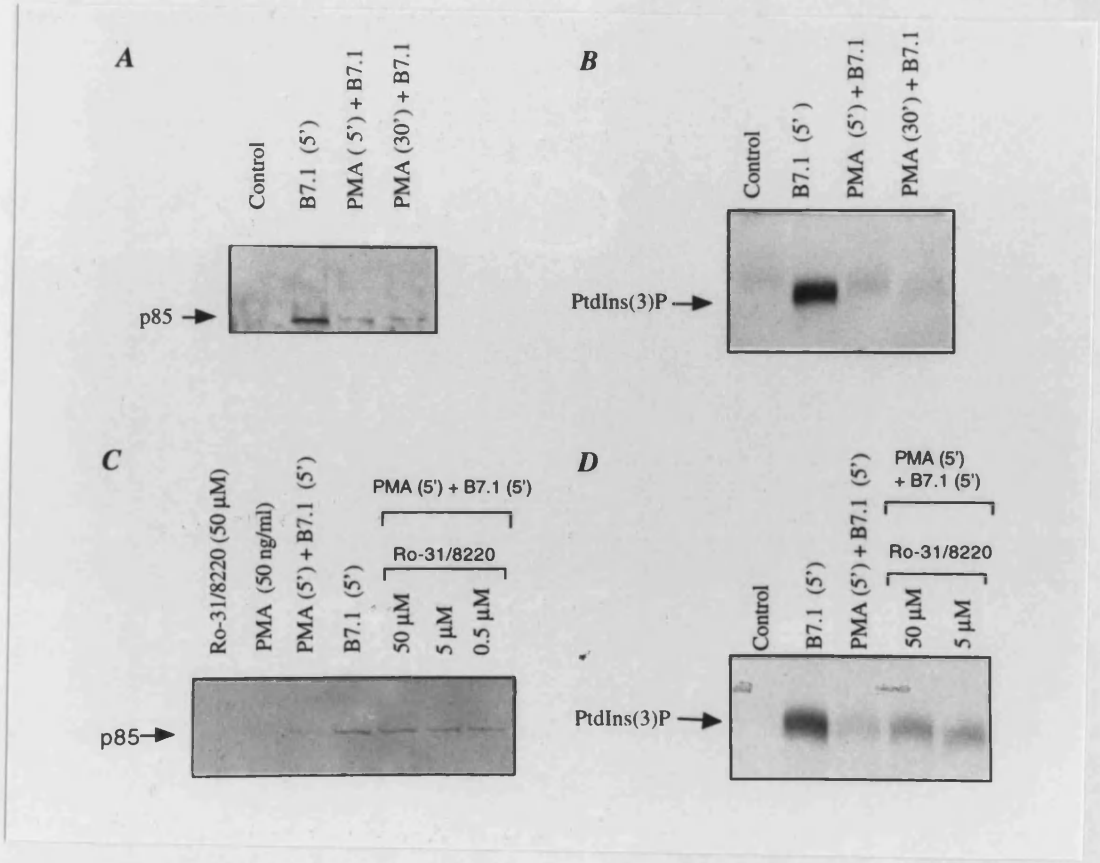


Figure 3.3.1 PMA disrupts CD28 coupling and activation of PI 3-kinase. **Panels A and C:** Western blots. Jurkat cells (2x 10^7/point) were pre-incubated as appropriate with PKC inhibitors prior to treatment with PMA, as shown above. Cells were then co-sedimented with CHO-B7.1$^+$ cells (10^7/point), before lysis in ice-cold NP40 lysis buffer. CD28 immunoprecipitates were prepared using the 9.3 mAb. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes. PI 3-kinase association was assessed by immunoblotting for the presence of the p85 subunit. **Panels B and D:** *in vitro* lipid kinase assays. Jurkat cells (2x10^7/point) were pre-incubated as appropriate with PKC inhibitors, prior to incubation with PMA as shown above. Cells were then co-sedimented with CHO-B7.1$^+$ cells (10^7) before lysis with ice-cold NP40 lysis buffer. CD28 immunoprecipitates were prepared using the 9.3 mAb and associated kinase activity assayed by phosphorylation of exogenous PtdIns as described (see section 2.2.15). Phosphorylated substrate was resolved by TLC and visualised by autoradiography.

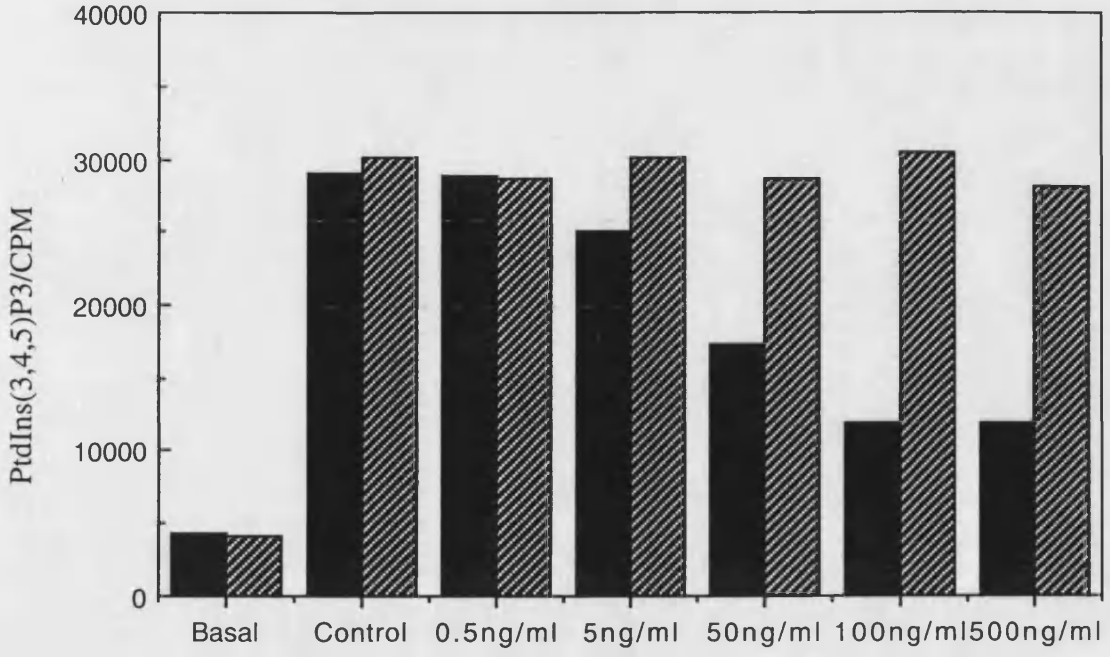


Figure 3.3.2a CD28 mediated PtdIns(3,4,5)P_3 accumulation in Jurkat cells is inhibited by PMA but not 4-α phorbol. Metabolically radiolabelled Jurkat cells ($2x10^7$) were pretreated with PMA (solid bars) or 4-α phorbol (hatched bars), at the concentrations described in the annotation, for fifteen minutes at 37°C. Cells were then co-sedimented with CHO-B7.1$^+$ cells for five minutes (except basal samples which were sedimented alone). Phospholipids were then extracted in chloroform, deacylated and analysed by HPLC as described (see section 2.2.14). Data expressed as absolute counts from single representative experiment.

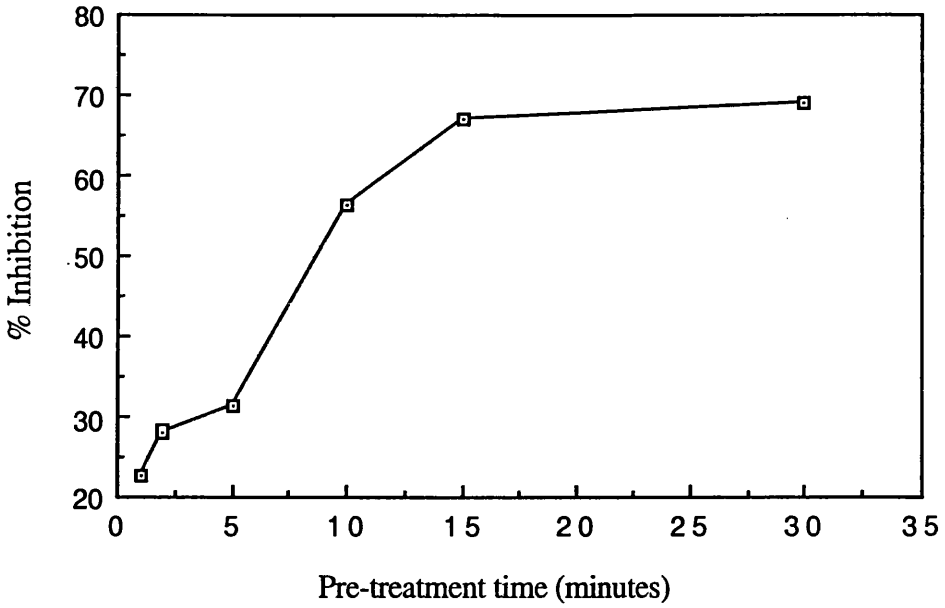


Figure 3.3.2b: Time course of PMA inhibition of CD28 mediated PtdIns(3,4,5)P_3 accumulation. Metabolically radiolabelled Jurkat cells ($2x10^7$) were pretreated with PMA (100 ng/ml) for times as shown above, prior to co-sedimentation with CHO-B7.1$^+$ cells ($1x10^7$). Phospholipids were then extracted in chloroform, deacylated and analysed by HPLC as described (see section 2.2.14). Results are expressed as inhibition of PI 3-kinase activity above basal.

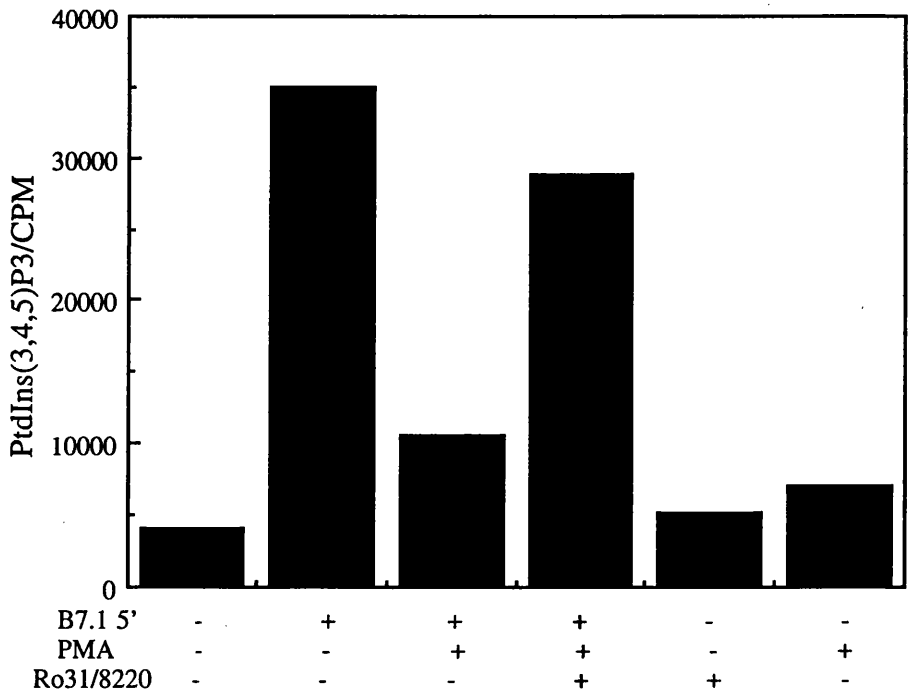


Figure 3.3.2c: PMA inhibition of CD28 mediated PtdIns(3,4,5)P_3 accumulation is reversed by an inhibitor of protein kinase C. Jurkat cells ($2x10^7$) were pretreated with Ro31/8220 (30μM), as shown, before pre-incubation with PMA (50 ng/ml) for fifteen minutes. Jurkat cells were then co-sedimented with CHO-B7.1$^+$ cells (10^7), at 37°C for five minutes. Following stimulation, phospholipids were extracted in chloroform, deacylated and analysed by HPLC as described (section 2.14). Data expressed as absolute counts from single representative experiment.

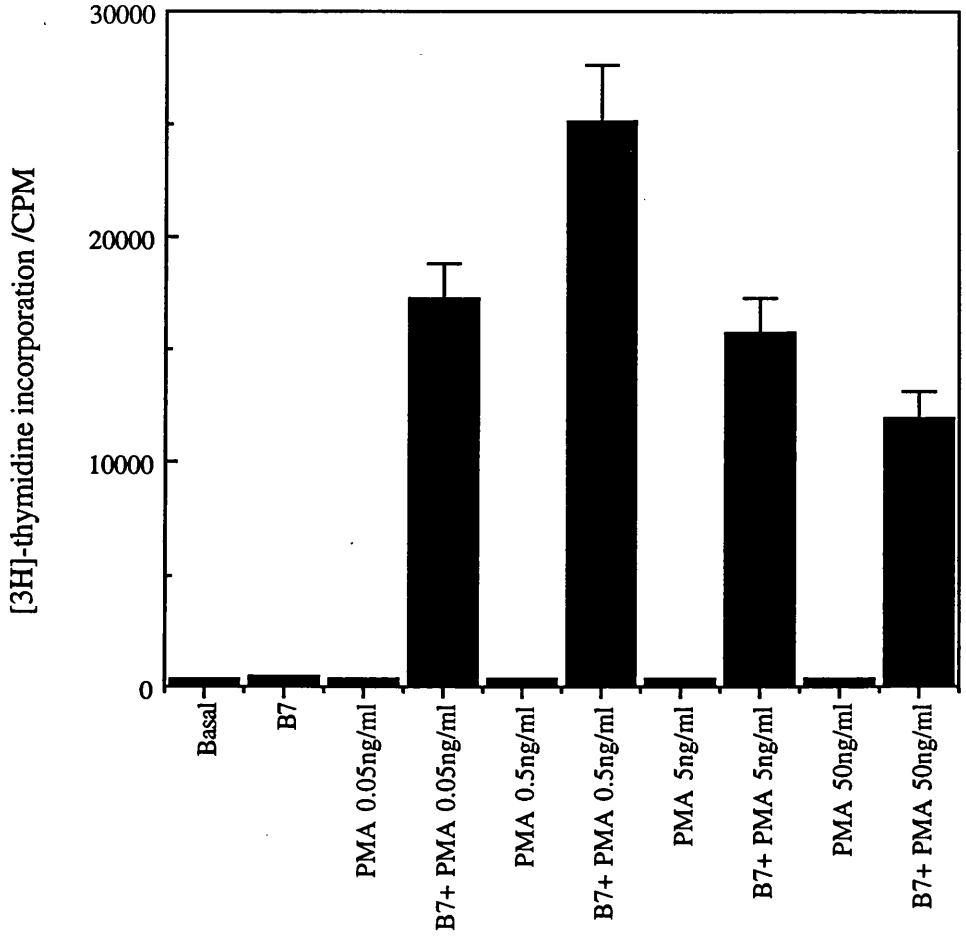


Figure 3.3.3a Effect of PMA on [^{3}H]-thymidine incorporation. Peripheral blood T cells (5x10^{4}cells/point) derived form normal healthy donors were treated as indicated. CHO-B7.1$^+$ cells were included, where indicated, at (2x10^4). Cells were harvested and assayed for [^{3}H]-thymidine incorporation as described (see section 2.2.21). Data are mean and standard error of quintuplicate replicates for a single representative experiment.

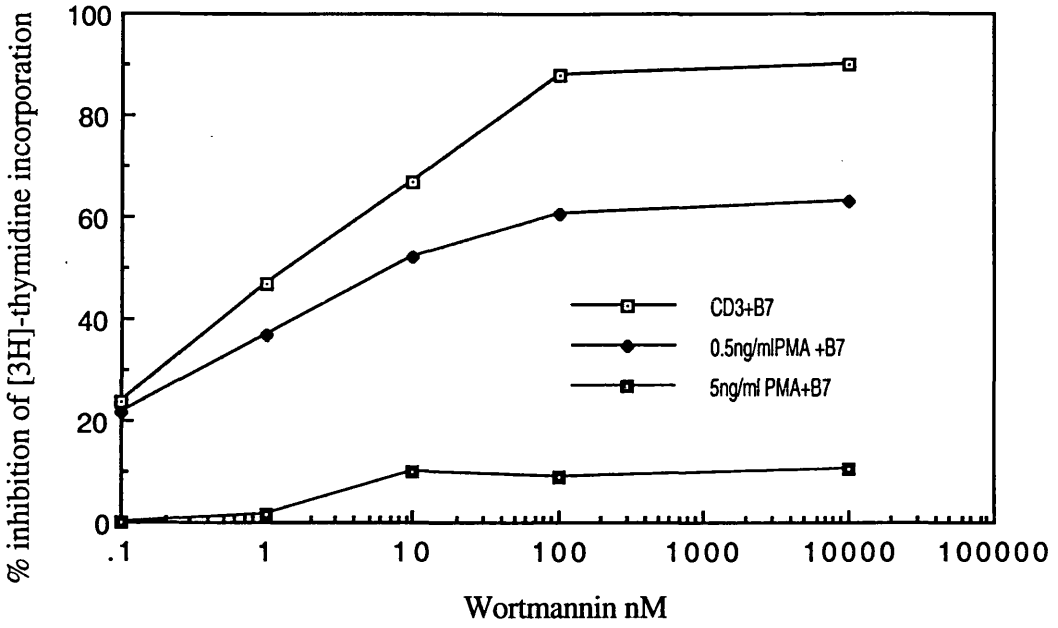


Figure 3.3.3b Effect of wortmannin on [^{3}H]-thymidine incorporation.

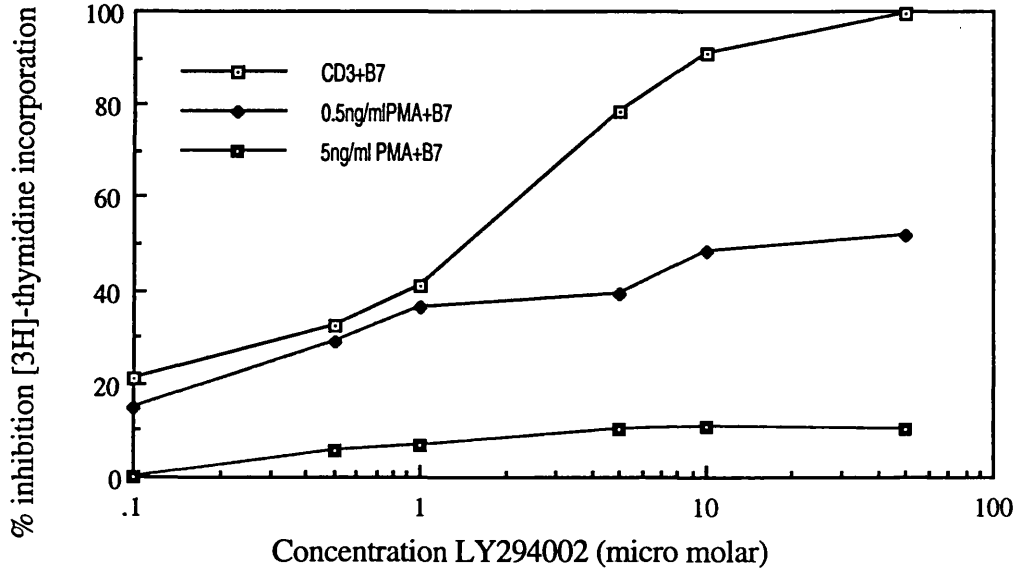


Figure 3.3.3c Effect of LY294002 on [^{3}H]-thymidine incorporation in purified primary T cells. Peripheral blood T cells ($5x10^4$) derived form normal healthy donors were treated as indicated or with anti-CD3 antibody UCHT1 (1 μg/ml) in the presence of cross linking antibody (1 μg/ml). CHO-B7.1$^+$ cells were included, where indicated, at ($2x10^4$). T cells were preincubated with wortmannin (Figure 3.3.3b) or LY294002 (Figure 3.3.3c) for 20 minutes prior to the addition of stimulatory agents. Cells were harvested and assayed for [^{3}H]-thymidine incorporation as described (see section 2.2.21). Results in b and c are expressed as percentage of inhibition of the thymidine incoporation induced by each treatment in the absence of wortmannin or LY294002. [^{3}H]-thymidine incorporation driven by CD3/B7, 0.5 ng/mlPMA/B7 and 5 ng/ml PMA/B7 were 69,929±4201 CPM, 55,100±3327 CPM and 40,260±3421 CPM respectively. Data are mean of quintuplicate replicates for a single representative experiment.

3.5 Summary.

1) CD28 ligation by B7.1 resulted in a rapid activation of PI 3-kinase. The subsequent accumulation of PtdIns(3,4,5)P_3 was maximal at an eight-fold increase over basal levels, after five minutes and followed by a slight decrease in lipid levels.

2) The accumulation of the lipid products of PI 3-kinase induced by B7.1 ligation of CD28, in Jurkat cells, was inhibited by wortmannin (100 nM), LY294002 (30 μM) and transient transfection of cells with Δp85.

3) The absence of lck does not inhibit the recruitment of PI 3-kinase to CD28 in JCaM1 cells. However, the subsequent activation of the enzyme, as demonstrated by the accumulation of its lipid product PtdIns(3,4,5)P_3, was severely impaired.

4) Pre-treatment of Jurkat T cells with PMA, inhibited CD28 association with the p85 subunit of PI 3-kinase after ligation by B7.1. Approximately 30% of stimulated PI 3-kinase activity, as assessed by the quantitation of its lipid products, was insensitive to the presence of the phorbol ester.

5) The effects of PMA on CD28 recruitment and activation of PI 3-kinase were not mimicked by the non-PKC activating phorbol ester 4-α phorbol, and were prevented by pretreatment of cells with the PKC inhibitor Ro31/8220.

6) PMA treatment of normal T cells in combination with B7 was sufficient to stimulate proliferation as measured by incorporation of [^{3}H]-thymidine. Proliferation was maximal at PMA concentrations of 0.5 ng/ml whilst higher concentrations still induced reduced proliferative responses.

7) Proliferation of normal T cells induced by CD3 and CD28 stimulation of cells was completely inhibited by inhibitors of PI 3-kinase wortmannin and LY294002. Proliferation of normal T cells induced PMA (0.5 ng/ml) in combination with B7 was partially inhibited by wortmannin and LY294002, whilst the reduced proliferative responses induced by higher concentrations of PMA (5 ng/ml) in combination with B7 were increasingly resistant to the effects of these PI 3-kinase inhibitors.

SECTION FOUR

Ligation stimulated phosphorylation of CD28

4.1 Phosphorylation of CD28 following stimulation by B7.1.

Ligation of CD28, by monoclonal antibody, has previously been reported to be followed by the phosphorylation of ^{173}Y within its cytoplasmic tail, which allows for the recruitment of PI 3-kinase [Pages *et al.* (1994); Prasad *et al.* (1994)]. Significant differences have been reported however, between CD28 mediated signals transduced following ligation by antibody or the natural ligand B7 [Nunes *et al.* (1994)]. Initial attempts to demonstrate the ligation dependent tyrosine phosphorylation of CD28 following ligation by B7.1 using Western blotting however, yielded inconclusive data, thus a metabolic labelling approach was adopted, in which CD28 was immunoprecipitated from Jurkat cells previously radiolabelled with ^{32}P inorganic phosphate. This procedure revealed the major component of CD28 phosphorylation to reside within serine and threonine residues. Since the serine/threonine phosphorylation of CD28 is a ligation dependent event it may be assumed to carry a physiological relevance either regulating the association of CD28 with PI 3-kinase or facilitating the coupling of CD28 to further signalling pathways. Experiments were therefore conducted to determine the nature of CD28 serine/threonine phosphorylation with respect to structural requirements of CD28 and sensitivity to pharmacological inhibitors.

Concordant with previously published data for CD28 phosphorylation following antibody stimulation [Pages *et al.* (1994); Prasad *et al.* (1994)], stimulation of CD28 by B7.1, the natural ligand, induced phosphorylation of CD28 which displayed rapid and sustained kinetics between 1 and 10 minutes, followed by a small decrease at 15 minutes (Figure 4.1.1a). Phosphoaminoacid analysis of the phosphorylated CD28, subsequent to ligation by B7.1 however, revealed the molecule to be heavily phosphorylated on serine and threonine residues, whilst tyrosine phosphorylation represented only a minor component of total phosphorylated residues (Figure 4.1.1b). This ligation-dependent phosphorylation was found to be specific to B7 ligation of CD28 since treatment of cells with CHO cells, UCHT1 mAb or phorbol ester, did not induce any detectable CD28 phosphorylation (Figure 4.1.1c)

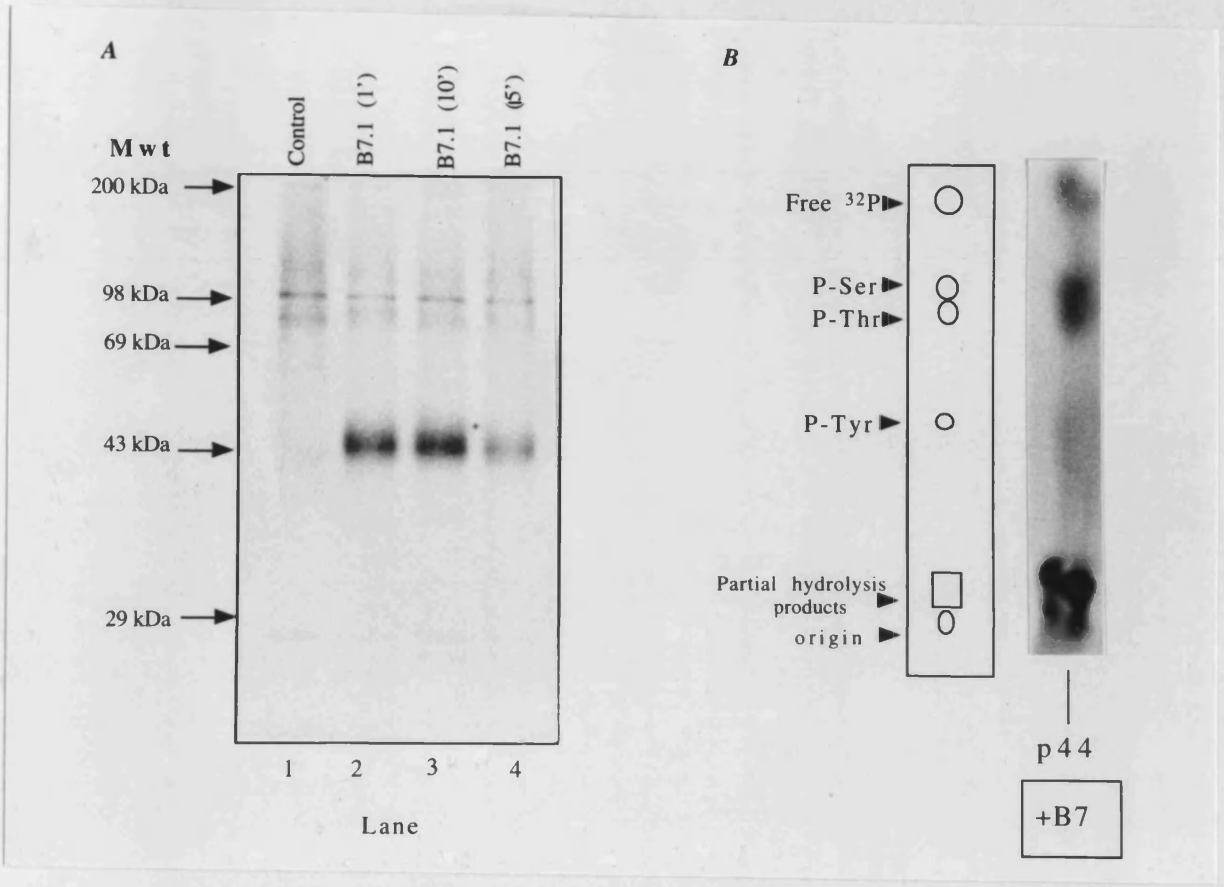


Figure 4.1.1a and b: B7.1 ligation of CD28 is followed by phosphorylation, predominantly on serine/threonine residues. Panel a: [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were co-sedimented with CHO B7.1$^+$ cells (10^7) and incubated at 37^{o}C. Cells were lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then resolved by SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9) and transferred to PVDF membrane as described (section 2.2.10). Phosphorylated proteins were located on the membrane by overnight autoradiography. Panel b: The appropriate portion of the PVDF membrane was excised, bound proteins hydrolysed and phosphoamino acids were separated as described (section 2.2.12) and visualised by autoradiography.

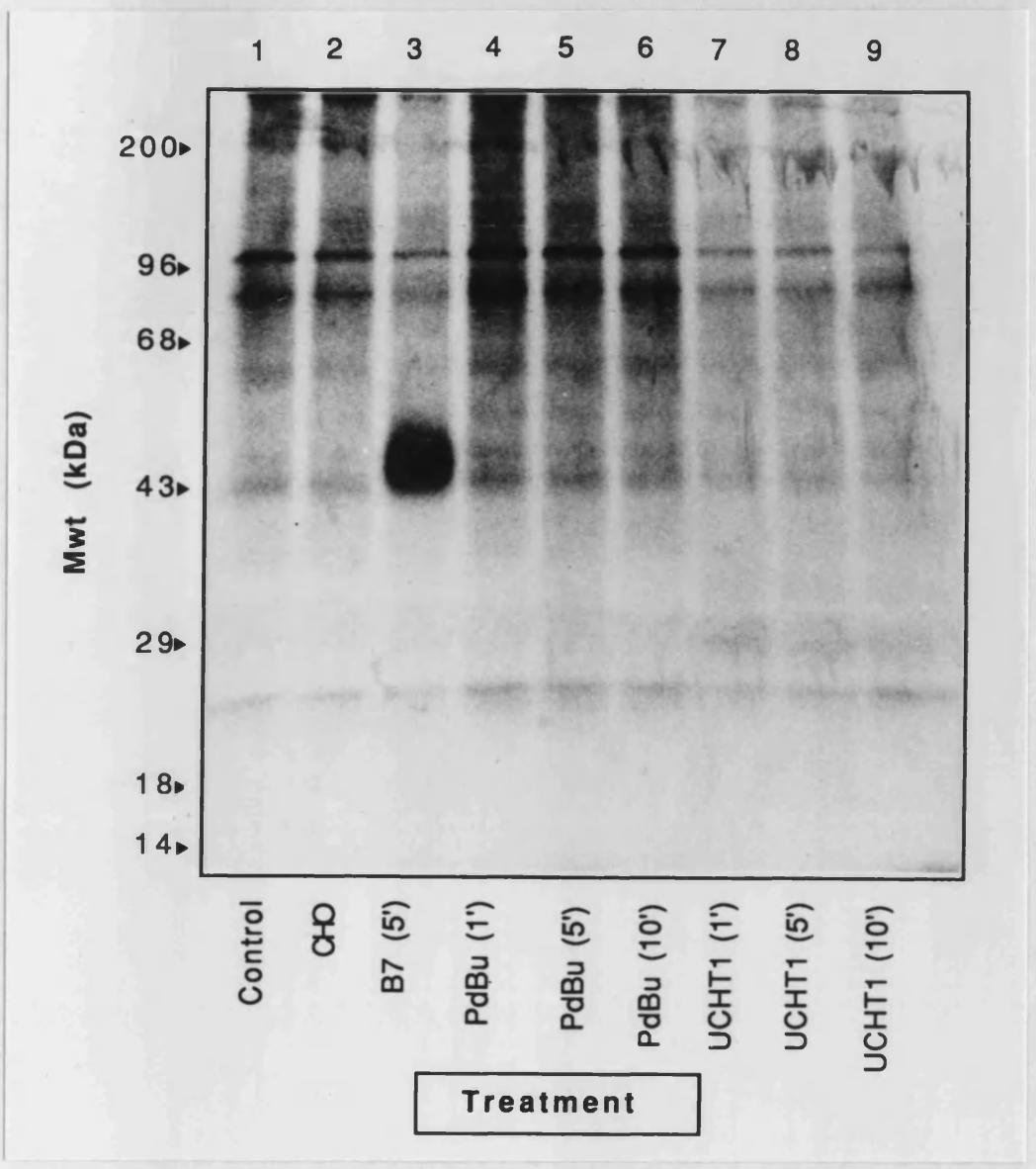


Figure 4.1.1c CD28 phosphorylation is specifically induced by CHO-B7.1 treatment of Jurkat cells. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were co-sedimented with CHO cells (10^7), CHO-B7.1$^+$ cells (10^7) or treated with PMA (50 ng/ml) or UCHT1 mAb (10 μg/ml) and incubated at 37^{o}C for the times indictaed. Cells were lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then resolved by SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9). Phosphorylated proteins were visualised by autoradiography at -80^{o}C.

4.2 The role of protein kinase C in mediating ligation-dependent phosphorylation of CD28.

The presence of three potential sites for phosphorylation by the serine/threonine protein kinase, protein kinase C, within the cytoplasmic domain of CD28 around residues ^{163}Ser, ^{177}Thr and ^{184}Thr strongly suggested a role for PKC in ligation stimulated CD28 phosphorylation. Moreover, PKC has previously been implicated in the threonine phosphorylation of CD28 [Hutchcroft *et al.* (1996)]. In order to investigate the role of PKC in CD28 phosphorylation therefore, [^{32}P]-Pi radiolabelled Jurkat cells were preincubated with 50 and 100 ng/ml PMA for fifteen minutes, conditions known to affect the signal transduction capabilities of CD28 (section 3.3), prior to sedimentation with or without CHO-B7^{+} cells. Immunoprecipitation of CD28 (Figure 4.2.1), revealed that pretreatment with PMA did not result in the phosphorylation of unstimulated CD28, nor was the ligation-stimulated phosphorylation of CD28 potentiated by the presence of phorbol esters in Jurkat cells.

In an alternative approach used to attempt to demonstrate a role for PKC in CD28 phosphorylation, radiolabelled Jurkat cells were incubated with the PKC inhibitor Ro31/8220 for ten minutes prior to stimulation with B7.1, conditions demonstrated to protect against the inhibitory action of PMA on CD28 coupling and activation of PI 3-kinase (Figure 3.3.1 B and D). Inhibition of PKC by Ro31/8220 was found not to inhibit B7.1 stimulated ligation-dependent phosphorylation of CD28 (Figure 4.2.2). Moreover, further inhibitors of PKC function such as Calphostin C (0.001-1 μM) and Bisindolyl maleimide (0.001 -1 μM) were found to be without effect upon the phosphorylation profile of CD28 (summarised in Table 4.1). These data strongly suggest that PKC does not mediate CD28 ligation-dependent serine/threonine phosphorylation in Jurkat cells.

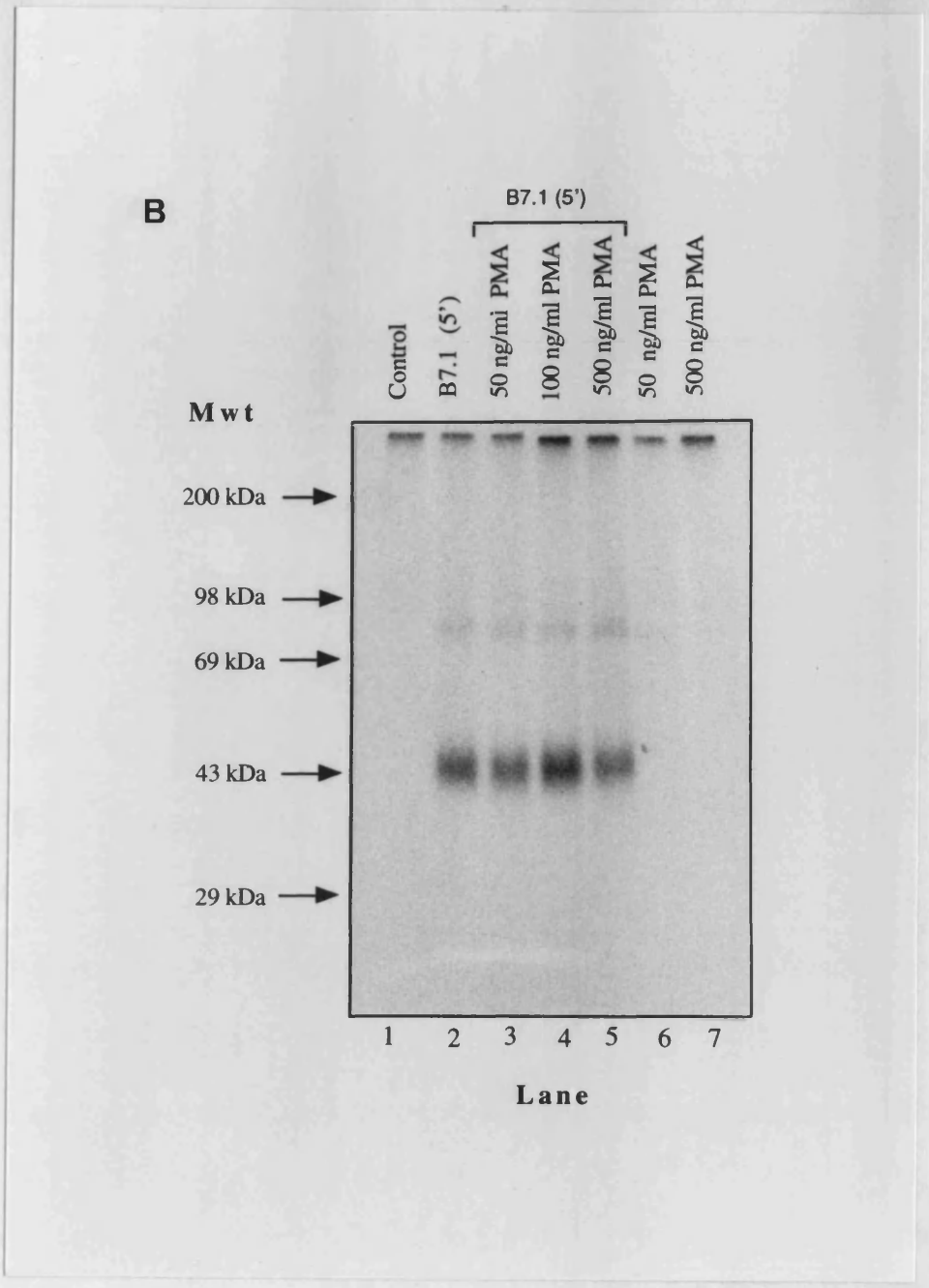


Figure 4.2.1 Effect of PMA on phosphorylation profile of CD28. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were pre-incubated with PMA (lanes 3 to 7) as indicated for 15 minutes prior to co-sedimentation with CHO B7.1$^+$ cells (10^7) (lanes 2 to 5) and incubated at 37^{o}C. Cells were lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins were visualised by autoradiography at -80^{o}C.

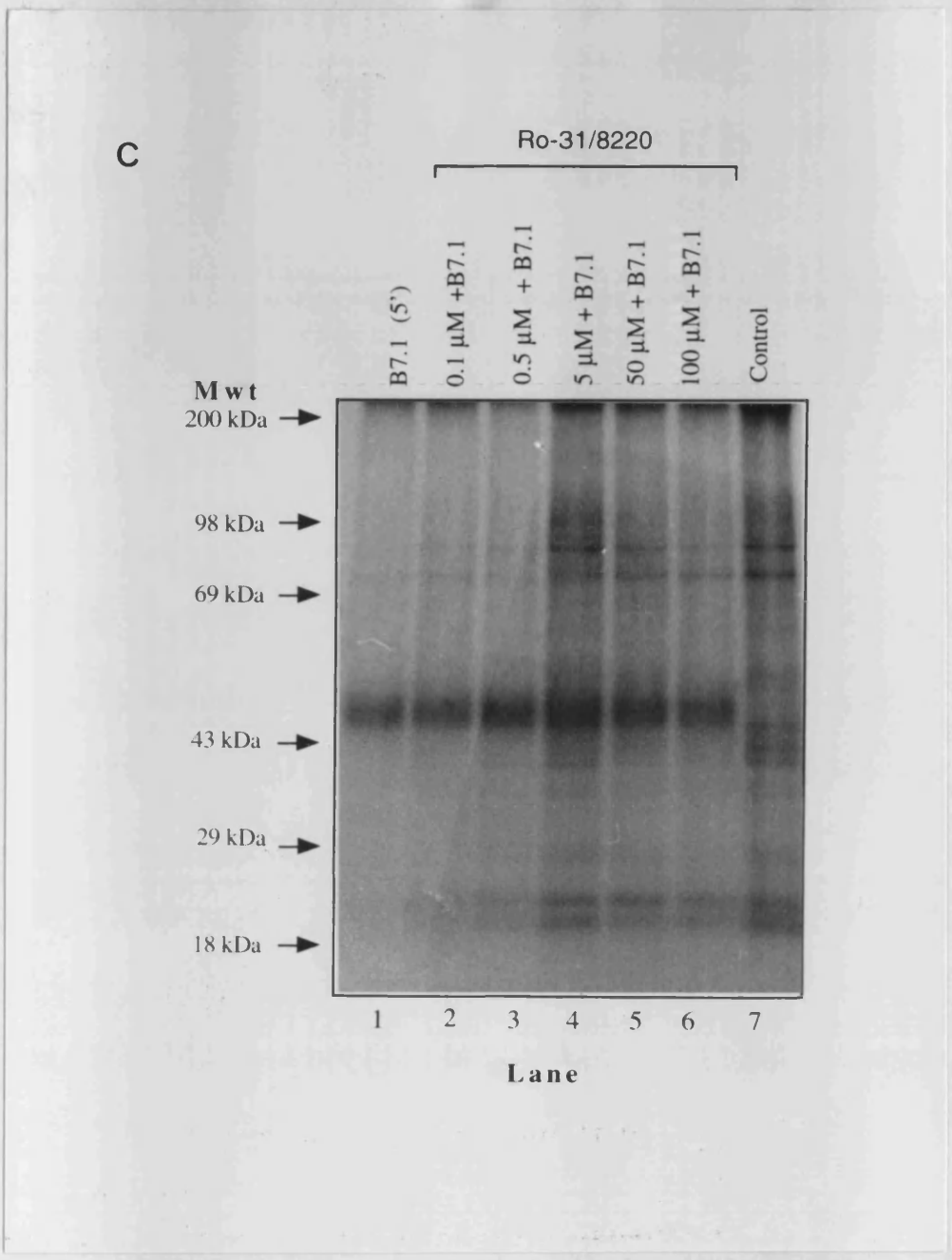


Figure 4.2.2 Effect of Ro31/8220 on ligation-dependent phosphorylation of CD28. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were pre-incubated with Ro31/8220 (lanes 2 to 6) for 10 minutes prior to co-sedimentation with CHO-B7.1$^+$ cells (10^7) (lanes 1 to 6) and incubation at 37^{o}C. Cells were lysed in NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins were visualised by autoradiography at -80^{o}C.

4.3 Effect of CD28 cytoplasmic tail truncation mutation on ligation stimulated phosphorylation.

To further characterise the nature of CD28 phosphorylation induced by B7.1 ligation, a murine T cell hybridoma DC27.1, stably transfected with wild type human CD28 [Pages *et al.* (1994)], or various cytoplasmic tail mutants which had been truncated by 21 (Δ 21) or 30 (Δ 30) C-terminal residues, were used. The cell surface expression of these CD28 mutants in DC27.1 cells was analysed by flow cytometry, and CD28 expression was found to be equivalent for each mutation. In murine T cell hybridomas transfected with human wild type CD28, B7.1 ligation resulted in the phosphorylation of CD28. In contrast to the phosphorylation observed in Jurkat, the ligation-induced phosphorylation of CD28 in DC27.1 cells was of shorter duration, such that phosphorylation was at a maximum after one minute post stimulation, and had declined to basal levels at ten minutes post stimulation (Figure 4.3.1). In T cell hybridomas transfected with del 21 CD28, B7.1 ligation induces phosphorylation, albeit to a lesser extent than that observed in wild type expressing cells (Figure 4.3.2). In cells expressing the CD28 del 30 truncation mutation however, B7.1 ligation did not induce any detectable phosphorylation of CD28 (Figure 4.3.2).

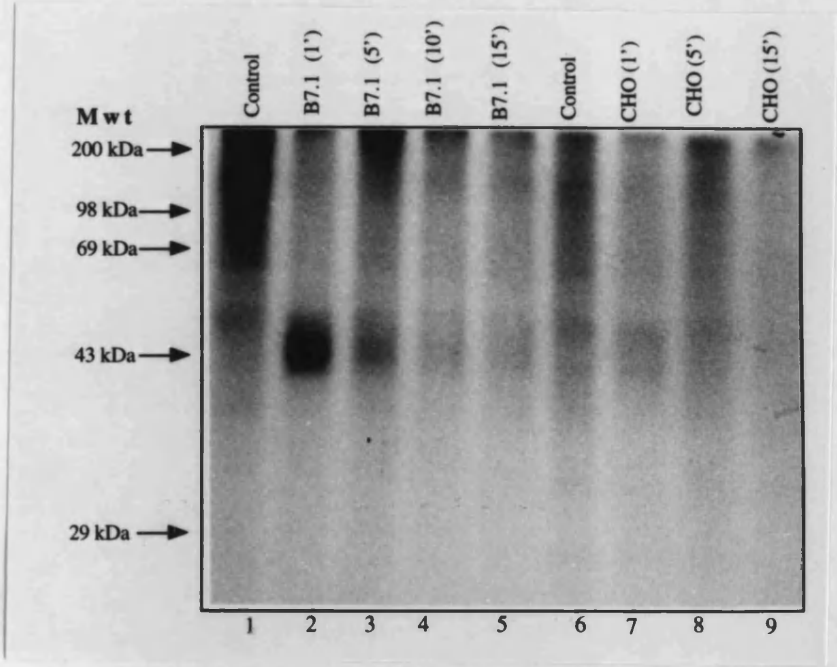


Figure 4.3.1 Ligation-dependent phosphorylation of human CD28 in murine DC27.1 cells. [^{32}P]-radiolabelled murine DC27.1 cells ($2x10^7$), expressing wild type CD28, were co-sedimented with CHO B7.1$^+$ cells (10^7) as indicated, and incubated at 37^{o}C. Cells were lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins visualised by autoradiography at -80^{o}C.

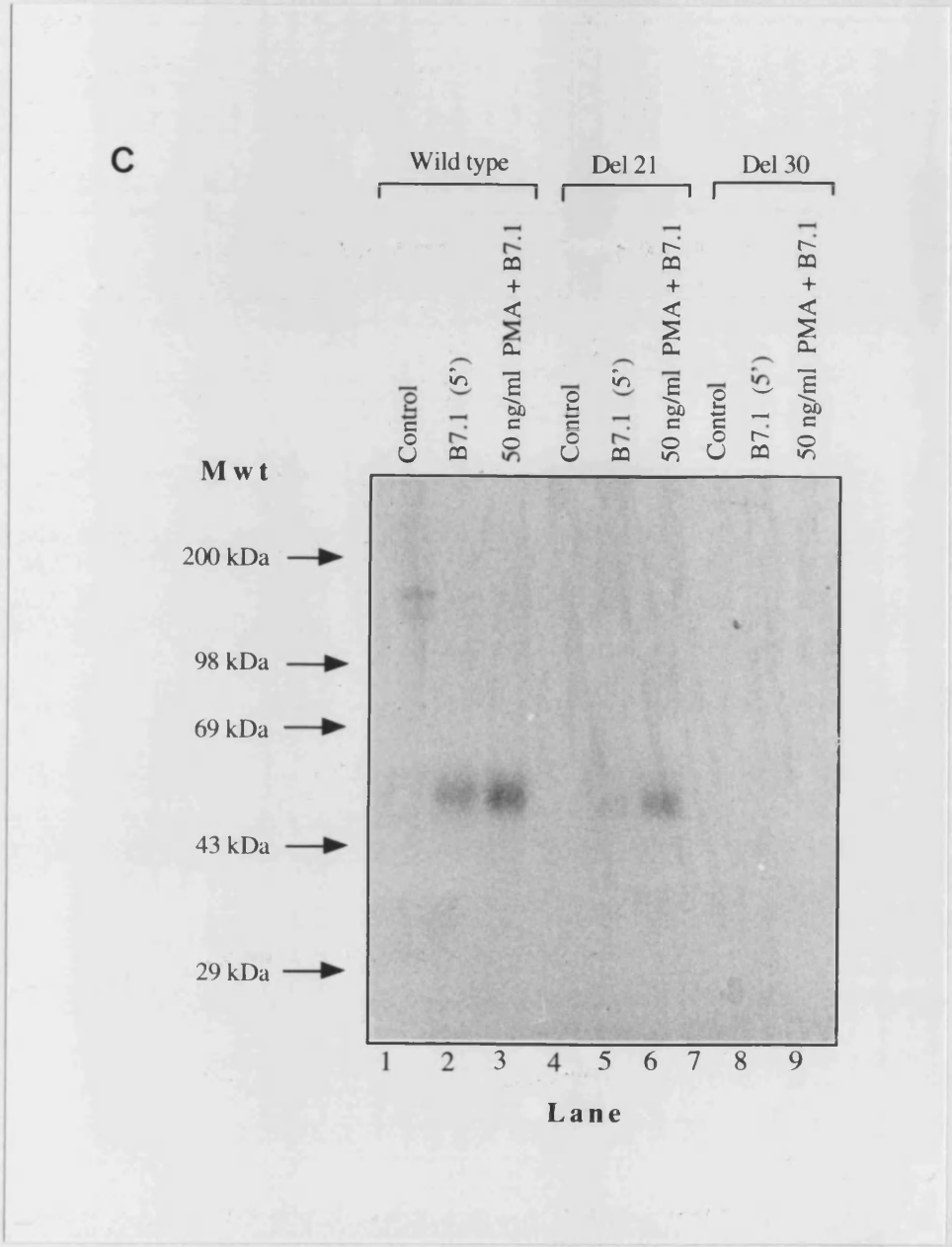


Figure 4.3.2 Effect of CD28 truncation mutations on ligation-dependent phosphorylation. [^{32}P]-radiolabelled murine DC27.1 cells ($2x10^7$), expressing wild type or truncated CD28 as indicated, were treated with PMA for 15 minutes, as indicated. Cells were then co-sedimented with CHO B7.1$^+$ cells (10^7), as indicated, and incubated at 37^{o}C for one minute. Cells were lysed in NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins were visualised by autoradiography at -80^{o}C.

4.4 Site mutagenesis of Tyr 173 prevents ligation-stimulated CD28 phosphorylation.

PI 3-kinase exhibits a unique dual specificity as both a lipid and protein serine/threonine kinase [Dhand *et al.* (1995)], and associates directly with CD28 (section 3.1) subsequent to CD28 ligation [Ward *et al.* (1996)]. It is, therefore, a possibility that PI

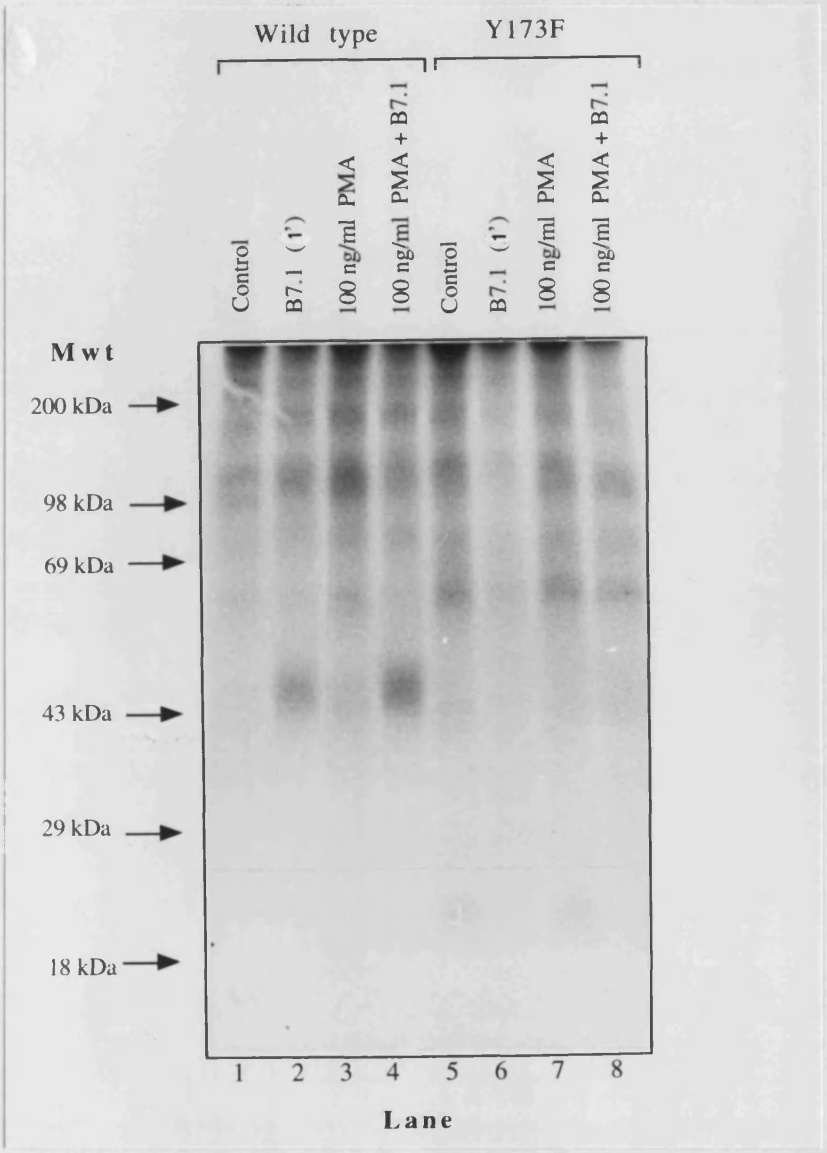


Figure 4.4.1 Effect of DYF173 point mutation on CD28 ligation dependent phosphorylation. [^{32}P]-radiolabelled murine DC27.1 cells ($2x10^7$/point) were co-sedimented with CHO-B7.1$^+$ cells (10^7 cells/point) (lanes 2, 4, 6 and 8) for one minute before lysis with NP40 lysis buffer. CD28 was immunoprecipitated with 9.3 mAb and immunoprecipitated proteins resolved by SDS-PAGE on a 7-17% acrylamide gradient gel. Phosphorylated proteins were then visualised by autoradiography.

3-kinase bound to the (p)^{173}YMNM motif may mediate ligation-stimulated phosphorylation of the CD28 cytoplasmic tail. To investigate this possibility, experiments were performed on the murine T cell hybridoma DC27.1 expressing either wild type human CD28 or a site-specific CD28 mutant in which ^{173}Tyr had been mutated to Phe (DYF173), since previous studies have reported that mutation of ^{173}Tyr is sufficient to completely abolish binding of PI 3-kinase to CD28 [Pages *et al.* (1994)]. In murine T cell hybridomas stably transfected with human wild type CD28, stimulation of CD28 by B7.1 resulted in the phosphorylation of CD28 (Figure 4.3.1). Interestingly, we were unable to detect ligation-stimulated phosphorylation of CD28 in DC27.1 cells expressing the DYF173 mutants, suggesting that the ligation dependent phosphorylation of CD28 is dependent upon the integrity of the YMNM motif.

4.5 Evidence that phosphorylation of CD28 on serine/threonine residues is independent of PI 3-kinase activity.

Since mutation of ^{173}Tyr has previously been shown to abolish CD28 coupling to PI 3-kinase [Pages *et al.* (1994)], the demonstration that engagement of B7.1 did not stimulate CD28 phosphorylation of the DYF173 mutant initially suggested the possible involvement of PI 3-kinase in modulating ligation-dependent CD28 serine/threonine phosphorylation. To further investigate this possibility, another site specific mutant CD28 was used in which ^{200}Tyr was mutated to Phe (DYF200) and stably expressed in the murine T cell hybridoma. Although ^{200}Tyr does not lie within a recognised consensus binding motif for the SH2 domains of the p85 subunit of PI 3-kinase, mutation of this residue has previously been reported to inhibit PI 3-kinase association with CD28 by approximately 90% [Pages *et al.* (1996)]. However, the precise role of ^{200}Tyr in the recruitment of PI 3-kinase to CD28 remains unclear. Given the observations that mutation of ^{173}Tyr disrupts both CD28 coupling to PI 3-kinase [Pages *et al.* (1994)] and ligation stimulated CD28 phosphorylation (Figure 4.4.1) it was surprising to note that mutation of ^{200}Tyr to Phe had no effect on the phosphorylation of CD28 after ligation by B7.1 (Figure 4.5.2).

The effects of wortmannin and the structurally unrelated PI 3-kinase inhibitor LY294002 on ligation-stimulated CD28 phosphorylation were also examined, since they act as inhibitors of both the protein kinase and lipid kinase activities of PI 3-kinase [Vlahos *et al.* (1994); Lam *et al.* (1994)]. However, concentrations of wortmannin and LY294002, which are known to inhibit CD28-stimulated PtdIns(3,4,5)P_3 accumulation, had no effect on phosphorylation of CD28 after ligation by B7.1 in Jurkat cells.

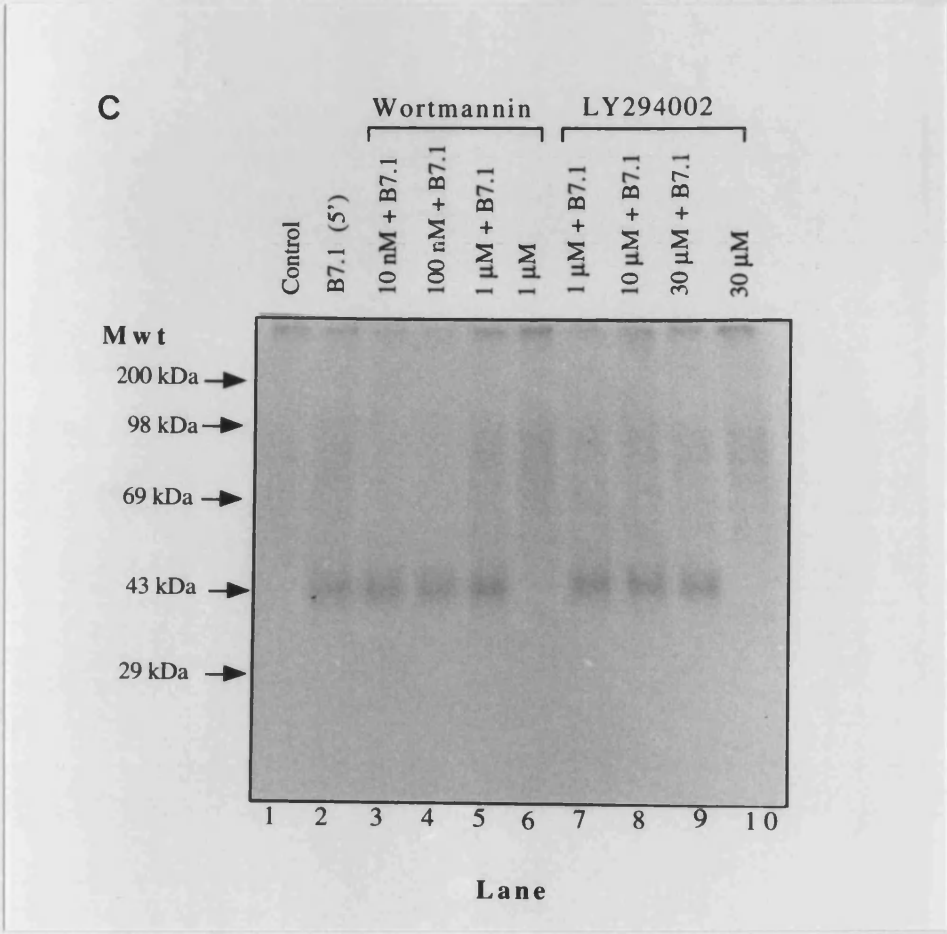


Figure 4.5.1 Ligation dependent phosphorylation of CD28 is resistant to inhibitors of PI 3-kinase. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were pre-incubated for ten minutes with wortmannin (lanes 3 to 6) or LY294002 (lanes 7 to 10), at the concentrations indicated. Cells were then co-sedimented with CHO-B7.1$^+$ cells (10^7) (lanes 2 to 5 and 7 to 9) and incubated at 37^{o}C for five minutes, followed by lysis in ice-cold NP40 lysis buffer. CD28 was immunoprecipitated with 9.3 mAb and proteins subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9). Phosphorylated proteins were visualised by autoradiography at -80^{o}C.

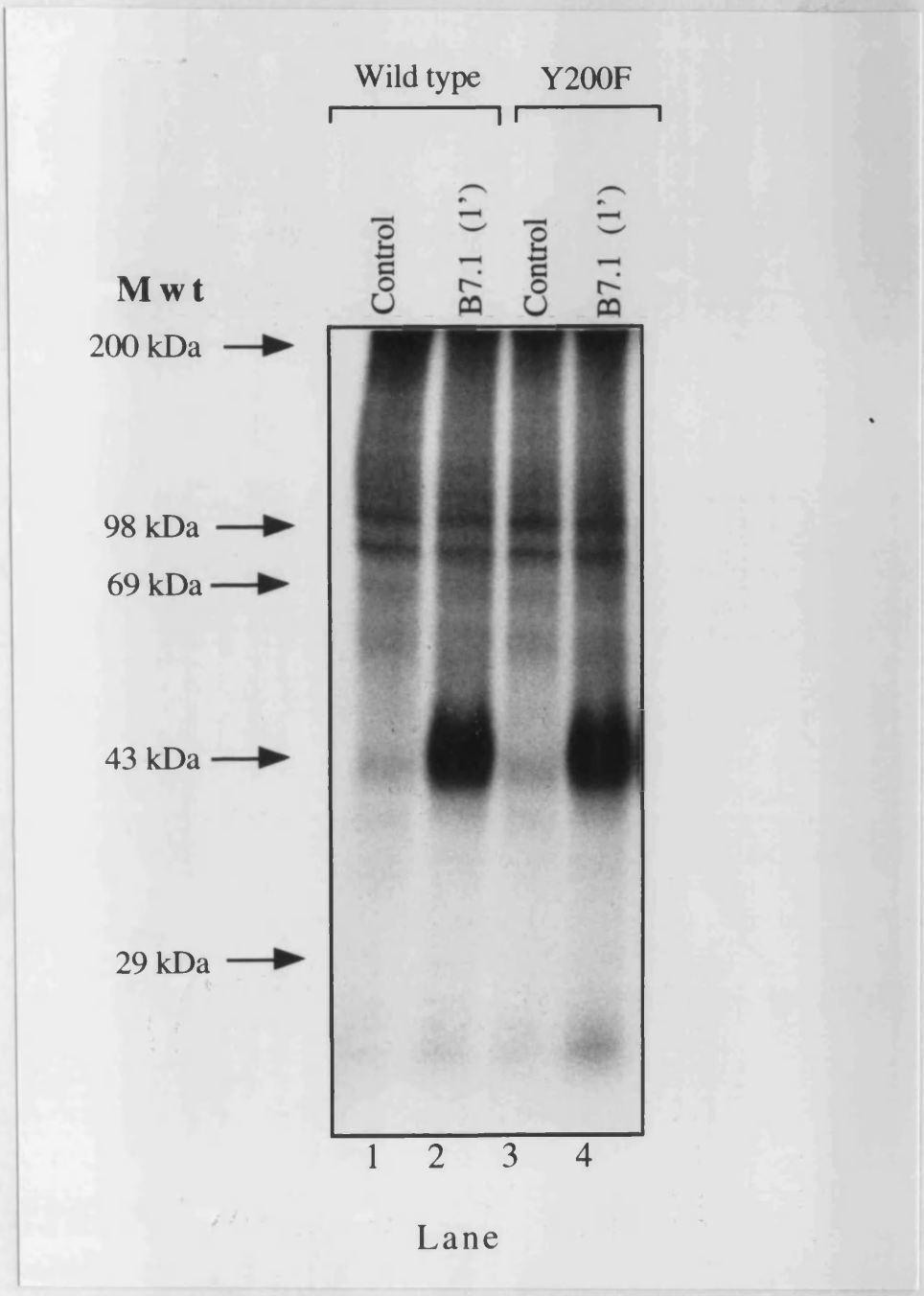


Figure 4.5.2 Effect of DYF200 point mutation on CD28 ligation dependent phosphorylation. [^{32}P]-radiolabelled murine DC27.1 cells (2x10^7/point) were co-sedimented with CHO-B7.1$^+$ cells (lanes 2 and 4) for one minute before lysis with NP40 lysis buffer. CD28 was immunoprecipitated with 9.3 mAb and immunoprecipitated proteins resolved by SDS-PAGE on a 7-17% acrylamide gradient gel. Phosphorylated proteins were then visualised by autoradiography -80^{o}C.

4.6 Effect of inhibitors or activators of protein serine/threonine kinases on the phosphorylation profile of CD28.

In a further attempt to elucidate the identity of the protein serine/threonine kinase that phosphorylates CD28 subsequent to B7.1 ligation, Jurkat cells were treated with 0.001-1 μM staurosporine [Davis *et al.* (1989)], 0.2-200 μM H8 [Hidaka *et al.* (1984)] or 0.5-50 μM calphostin C [Tamaoki (1991)], which are known to inhibit several kinases including PKC, cAMP-dependent protein kinase and/or cGMP-dependent protein kinase. Treatment of Jurkat cells with these inhibitors however, did not detectably modify the phosphorylation profile of CD28. Moreover, treatment of Jurkat cells with agents known to activate cAMP-dependent protein kinase such as 5 μM forskolin in combination with 500 μM isobutylmethylxanthine for one hour; or 0.1-100 μM 8-bromo-cAMP for 5-15 minutes and cGMP-dependent protein kinase such as 100 μM sodium nitroprusside or 0.1-100 mM 8-bromo-cGMP for 15 minutes, had no detectable effect on either basal or ligation-stimulated phosphorylation of CD28. The activators and inhibitors of serine/threonine kinases that were used without effect on ligation-stimulated phosphorylation of CD28, along with the concentrations and incubation times are summarised in Table 4.1.

One serine/threonine residue of the CD28 cytoplasmic domain lies within a potential site for phosphorylation by the proline-directed ERK serine/threonine kinases (namely ^{177}TPR). This is particularly interesting given the observation that truncation mutants lacking this site exhibited markedly impaired ligation-stimulated phosphorylation of CD28 (Figure 4.3.2), therefore, the effect of PD98059, which inhibits MAP kinase kinase (MEK) and thus ERK activation was studied. One hour pretreatment with PD98059 0.1-50 μM [Dudley *et al.* (1995)] however, had no effect on the phosphorylation of CD28 after B7.1 ligation (Figure 4.6.1). Similarly, SB203580 (30 μM) which inhibits the related, but distinct p38 MAP kinase [Cuenda *et al.* (1995)], also had no effect on ligation stimulated CD28 phosphorylation. The data is summarised in Table 4.1.

Ligation of CD28 has been demonstrated to result in the activation of the acidic form of sphingomyelinase [Boucher *et al.* (1995)], which elevates ceramide levels activating serine/threonine kinases including ceramide activated protein kinase (CAPK) [Matthias *et al.* (1991)], and PKCζ [Lozano *et al.* (1994)]. Therefore, experiments were performed to determine whether ceramide analogues could stimulate the phosphorylation of CD28. Accordingly, radiolabelled Jurkat cells were incubated for an

hour with active C2, (2S,3R)-D-*erythro*-*N*-Acetylsphingosine, and inactive C2H2 (DL-*erythro*-Dihydro-C_2-ceramide) cell permeable analogues of ceramide [Cifone *et al.* (1993); Westwick *et al.* (1995)], both at 10 and 50 μM (Figure 4.6.1). It was not possible to detect any CD28 phosphorylation induced by ceramide under these conditions. Furthermore, B7.1 stimulated ligation dependent phosphorylation of CD28 was found to be insensitive to the presence of chloroquine (20 μM to 2mM, an inhibitor of the activation of acidic sphingomyelinase [Boucher *et al.* (1995)] (Table 4.1). These data suggest that ceramide signalling is not responsible for the serine/threonine phosphorylation of CD28.

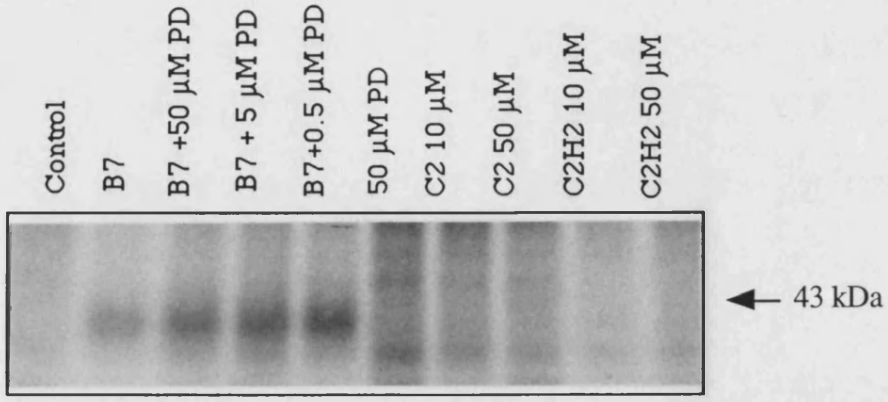


Figure 4.6.1 Effect of a MAP kinase inhibitor and ceramide analogues on phosphorylation profile of CD28. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were pre-incubated with agents indicated, where PD is PD98059, C2 is active ceramide and C2H2 is the inactive analogue. co-sedimention with CHO B7.1$^+$ cells (10^7), indicated as B7, and incubation at 37^{o}C. Cells were lysed in ice-cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins were visualised by autoradiography at -80^{o}C.

Table 4.1 Serine/threonine kinase inhibitors and activators without effect on phosphorylation profile of CD28.

Compound	Concentration (μM)	Incubation time (min)	Kinase targets
inhibitors			
Staurosporine	0.001-0.1	20	PKC, PKA, MLCK, Casein kinase 1, Ca^{2+}/CaM dependent kinase, PKG
Ro31/8220	0.5-50	5-10	PKC, Ca^{2+}/CaM dependent kinase, PKA
Calphostin C	0.5-50	20	PKC, PKA, PKG
H89	5-50 nM	10	PKA
H7	0.2-200	10	PKA, PKC, PKG, MLCK
PD98059	0.1-50	60	MEK
SB203580	0.3-30	60	p38
Chloroquine	0.02-2 mM	10	Acidic sphingomyelinase
activators			
Forskolin	5	30-60	PKA
Forskolin/IBMX	5/500	60	PKA
8-bromo-cAMP	100	5-15	PKA
Rp-cAMPs	0.5-1	15	PKA
8-bromo-cGMP	100	5-15	PKG
Na nitroprusside	0.2-20	5-15	PKG

4.7 Role of protein tyrosine kinases in ligation-stimulated phosphorylation of CD28.

Since previous results had suggested that the integrity of ^{173}Tyr residue, within the CD28 cytoplasmic tail, was a prerequisite to serine/threonine phosphorylation of CD28 (Figure 4.4.1), an experiment was performed to determine if inhibition of protein tyrosine kinase activity also inhibited ligation-dependent phosphorylation of CD28. Jurkat cells were incubated overnight (16 hours) with or without Herbimycin A (1 μM), prior to metabolic labelling with ^{32}P inorganic phosphate. Cells were then stimulated by co-sedimentation with CHO-B7.1^{+} cells, and CD28 immunoprecipitated with 9.3 mAb. CD28 phosphorylation was found to be markedly reduced in cells that had been treated with Herbimycin A (Figure 4.7.1) compared to vehicle treated cells. Difficulties arise in the interpretation of this experiment however since the observed inhibition of phosphorylation may be due to a non-specific effect of Herbimycin on the metabolism of treated cells, this is discussed further in section 7.2.1.

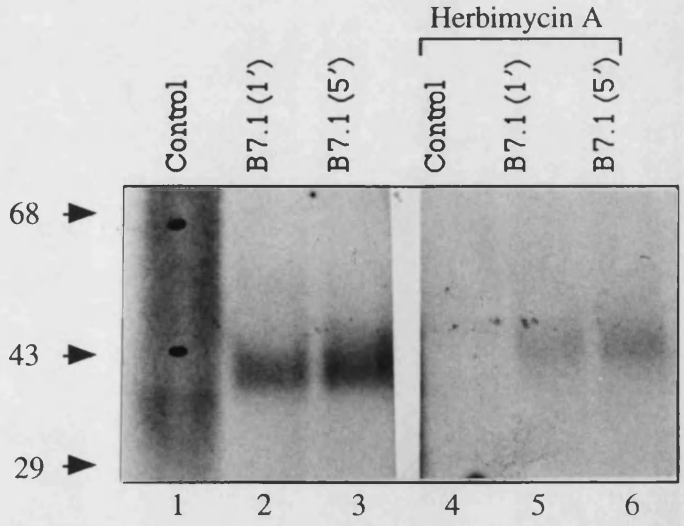


Figure 4.7.1 Effect of herbimycin A on ligation-dependent phosphorylation of CD28. Jurkat cells were incubated with (lanes 4 to 6) or without (lanes 1 to 3) Herbimycin A (1 μM) overnight. Cells were then metabolically radio-labelled with [^{32}P] as described (section 2.2.11). Radiolabelled cells ($2x10^7$) were then co-sedimented with CHO-B7.1^{+} cells (10^7) and incubated at 37^{o}C. Cells were lysed in NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Immunoprecipitated proteins were then subjected to SDS-PAGE using a 7-17% acrylamide gradient gel (section 2.2.9), and phosphorylated proteins visualised by autoradiography.

4.8 Summary.

1) Ligation of CD28 is followed by heavy phosphorylation of its cytoplasmic serine and threonine residues, whilst tyrosine phosphorylation represents only a minor component of the detectable phosphorylated amino acid residues.

2) Despite the presence of several consensus site for phosphoryation by PKC within the cytoplasmic domain of CD28, no phosphorylation was induced by PMA and ligation-dependent phosphorylation of CD28 was found to be insensitive to inhibition of PKC.

3) Phosphorylation of CD28 requires the integrity of the ^{173}YMNM motif since site-specific mutagenesis or truncation mutation of ^{173}Y severely abrogated ligation-dependent phosphorylation.

4) Ligation dependent phosphorylation of CD28 occurs independently of the protein or lipid kinase function of PI 3-kinase since inhibitors of PI 3-kinase did not inhibit this response, and site specific mutagenesis of ^{200}Y, which prevents PI 3-kinase association with CD28, was without effect on CD28 ligation-stimulated phosphorylation.

5) Studies with a range of pharmacological inhibitors indicate that ligation-dependent phosphorylation of CD28 occurs independently of PKA, PKG, as well as ERK and p38 MAP kinase and ceramide generated signals.

SECTION FIVE

CD28 activation of downstream effectors and regulators of PI 3-kinase-dependent signalling cascade

5.1. CD28 mediates activation of protein kinase B.

The demonstration that ligation of CD28 resulted in the recruitment and strong activation of PI 3-kinase, suggested that CD28-mediated activation of PI 3-kinase may be sufficient to induce activation of downstream effector molecules of PI 3-kinase. Additionally, the demonstration that ligation of CD28 was followed by heavy phosphorylation of CD28 on serine and threonine residues (as discussed in section four) revealed that CD28 ligation by B7.1 is followed by activation of serine/threonine kinases. The recently identified serine/threonine kinase PKB has been implicated as a downstream effector of PI 3-kinase by a number of lines of evidence (discussed in section 1.5.6.1.2). Furthermore, since activation of PKB has been widely reported as protective against apoptosis [Dudek *et al.* (1997); Kulik *et al.* (1997); Kwhaja *et al.* (1997); Hausler *et al.* (1998)], activation of PKB would fit well as a downstream effector of CD28. Accordingly, a number of experiments were performed to examine the effects of CD28 ligation on the activity of PKB.

5.1.1 PKB activation by constitutively active PI 3-kinase in Jurkat cells.

To demonstrate the expression of CD28 and/or rCD2 on transiently transfected cells and the effectiveness of both the transfection and the PKB assay, Jurkat cells were transfected with plasmid DNA for HA-PKB and the constitutively active PI 3-kinase chimaeric construct which consisted of the rCD2 extracellular and transmembrane domain linked to p110 or a kinase dead version of this construct, rCD2p110R/P. The cell viability after transfection was found by trypan blue exclusion after transfection to be consistent at approximately 40% viability for each transfection condition. Flow cytometric immunofluorescence analysis of the viable Jurkat cells 16 hours after transfection confirmed expression of CD28 on Jurkat cells transfected with either HA-PKB alone or with rCD2p110 or rCD2p110R/P (Figure 5.1.1a). Furthermore, cell surface expression of the different rCD2-PI 3-kinase chimaeras was confirmed by flow cytometric immunofluorescence with the anti-rCD2 mAb Ox34 (Figure 5.1.1a). Considerable amounts of PKB activity were detected in the anti-HA immunoprecipitates derived from

Jurkat cells co-transfected with HA-PKB and rCD2p110, compared to cells transfected with HA-PKB alone (Figure 5.1.1b). Whilst some PKB activity was detected in anti-HA immunoprecipitates derived from Jurkat cells co-transfected with the kinase dead rCD2p110R/P construct (which contains an inactivating arginine to proline mutation at position 1130) and HA-PKB, this was considerably less than that detected in cells co-transfected with rCD2p110 and HA-PKB (Figure 5.1.1b). Since cells transfected with HA-PKB alone exhibit some basal PKB activity in HA-PKB immunoprecipitates (Figure 5.1.1b), the increased expression of HA-PKB in cells co-transfected with rCD2p110R/P (Figure 5.1.1b middle panel). Additionally, there appears to be more protein immunoprecipitated in the rCD2p110R/P lane (Figure 5.1.1b upper panel). These observations may explain the increased PKB activity of cells co-transfected with rCD2p110R/P and HA-PKB.

5.1.2 Activation of PKB in Jurkat cells following CD28 ligation.

To assess the effects of CD28 ligation on PKB activation in T cells, cells of the leukaemic T cell line Jurkat that had been transfected with HA-tagged PKB were intially used. Ligation of CD28 by either its natural ligand B7.1 or the anti CD28 mAb 9.3 resulted in the rapid and transient activation of PKB in transiently transfected Jurkat cells (Figure 5.1.2a). Moreover, the activation of PKB following CD28 ligation by B7.1 could be prevented by pretreatment of the Jurkat cells with the PI 3-kinase inhibitors wortmannin and LY294002 (Figure 5.1.2.a/b). Although parental CHO cells increased PKB activity above that seen under basal unstimulated conditions this was not time dependent and only partially sensitive to wortmannin (Figure 5.1.2a). It is nevertheless surprising to note that wortmannin suppresses the PKB activity stimulated by B7.1 ligation of CD28 to a level that is much lower than the observed PKB activation by parental CHO. Immunoblotting revealed that this observation is unlikely to be explained by discrepancies in HA-PKB expression and the reasons for this observation remain unclear. Since the histone H2B substrate used to detect PKB activity can be phosphorylated by many protein kinases in cell extracts [Burgering and Coffer (1995); Franke *et al.* (1995); Alessi *et al.* (1997)], this observation may simply reflect discrepancies in the washing of the individual immunoprecipitates, and hence the amount of kinase activity non-specifically associated with the protein G-sepharose beads.

Figure 1A

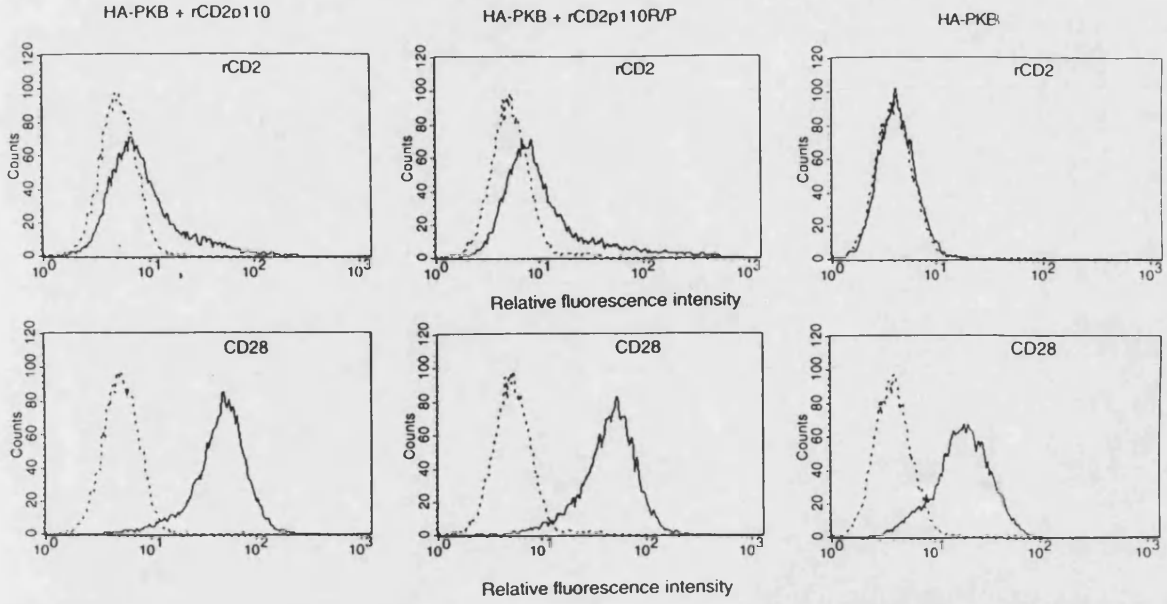
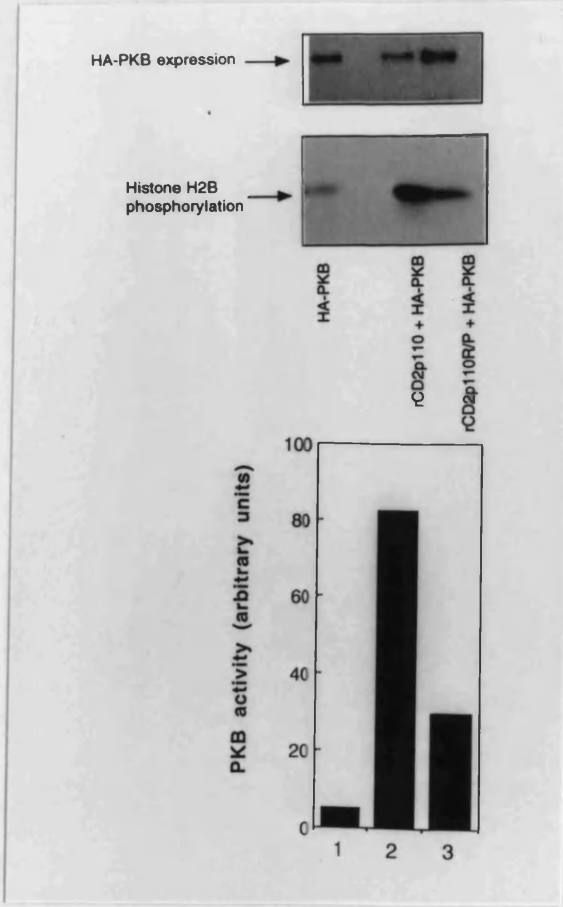


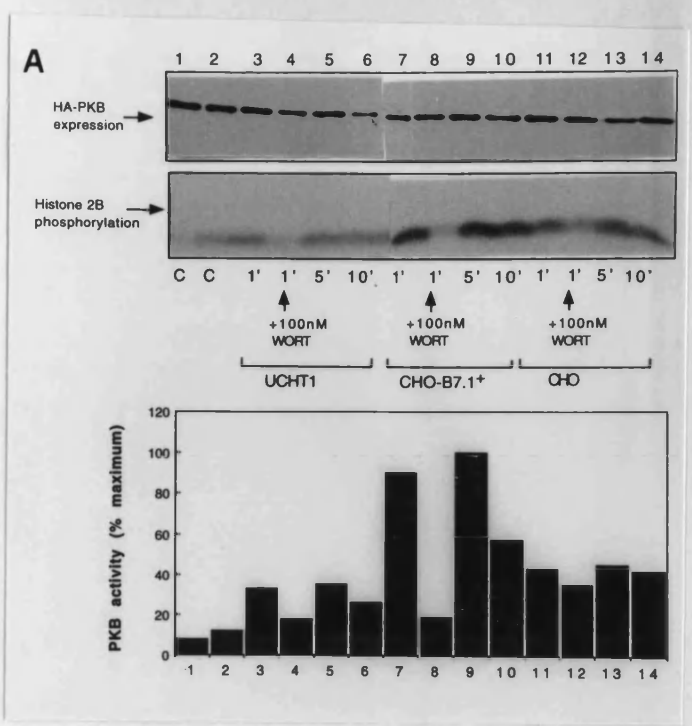
Figure 5.1.1 a Flow cytometry of transiently transfected Jurkat cells for expression of CD28, rCD2p110 and rCDp110R/P and activation of PKB by PI 3-kinase. (a) Jurkat cells which had been transiently transfected with HA-PKB, rCD2p110 or rCD2p110R/P were stained with either a control mouse IgG (dotted lines), or anti-CD28 mAb 9.3 or the anti-rCD2 Ox34 (solid lines) as indicated. (b) Jurkat cells (10^7/point) which had been transiently transfected with HA-PKB or co-transfected with either rCD2p110 or rCD2p110R/P were lysed and immunoprecipitates prepared with anti-HA mAb. The immunoprecipitates were subjected to a PKB assay using histone H2B as a substrate. The reactions were stopped and proteins resolved by SDS-PAGE. The upper part of the gel was transferred to PVDF and immunoblotted with anti-PKBα polyclonal antibody (upper panel). The lower part of the gel was dried and histone H2B phosphorylation was detected by autoradiography at -80°C (middle panel) followed by densitometric analysis (lower panel). Data are from single representative experiment.

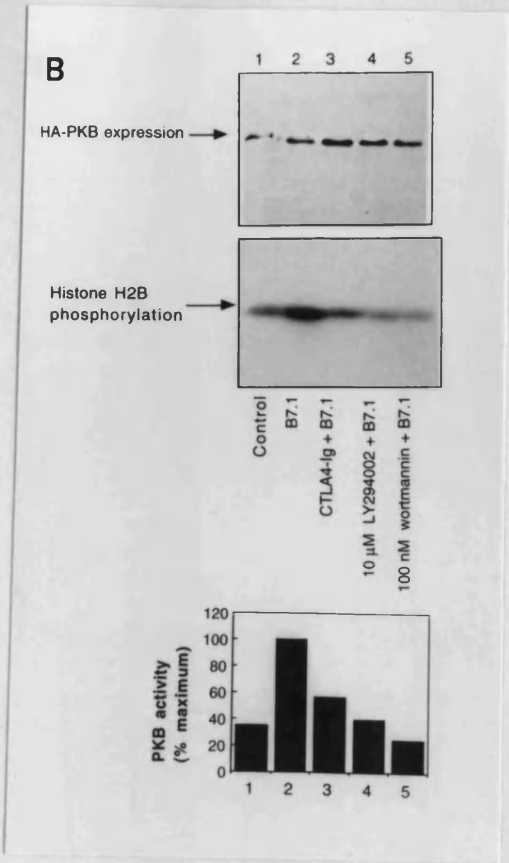


The specificity of the CD28 stimulated PKB activation which was observed after ligation by B7.1 was investigated using the CTLA4-Ig fusion protein. Incubation of CHO-B7.1$^+$ cells with CTLA4-Ig fusion protein prior to co-sedimentation with Jurkat cells also prevented the activation of PKB (Figure 5.1.2b). No PKB activity was detectable in either untreated control or CD28-activated Jurkat cells which had been transfected with the plasmid DNA for HA-PKB(-) (Figure 5.1.2c). Expression of HA-PKB or HA-PKB(-) and efficiency of immunoprecipitation was confirmed by immunoblotting of the immunoprecipitates with anti-PKBα Ab (Figure 5.1.2b upper panel).

5.1.3 Activation of PKB in normal T lymphocytes by CD28.

Given that the signalling pathways relating to CD28 activation in normal T lymphocytes are still poorly defined, and that the signalling mechanisms observed in cell lines do not necessarily faithfully reflect those which operate in normal in normal T lymphocytes, it was investigated whether or not ligation of CD28 in freshly isolated primary T cells also resulted in the activation of PKB. For these studies, endogenous PKB was immunoprecipitated using an anti-PKBα polyclonal Ab. Accordingly, endogenous PKB activity present in anti-PKB immunoprecipitates derived from normal T lymphocytes stimulated with anti-CD28 mAb 9.3 was enhanced above that observed under resting conditions (Figure 5.1.3). Moreover, the increase was inhibited by pretreatment of the cells with the PI 3-kinase inhibitor wortmannin (Figure 5.1.3). Immunoblotting analysis with anti-PKB Ab at 1:1000 dilution confirmed the efficiency of immunoprecipitation (Figure 5.1.3 upper panel).





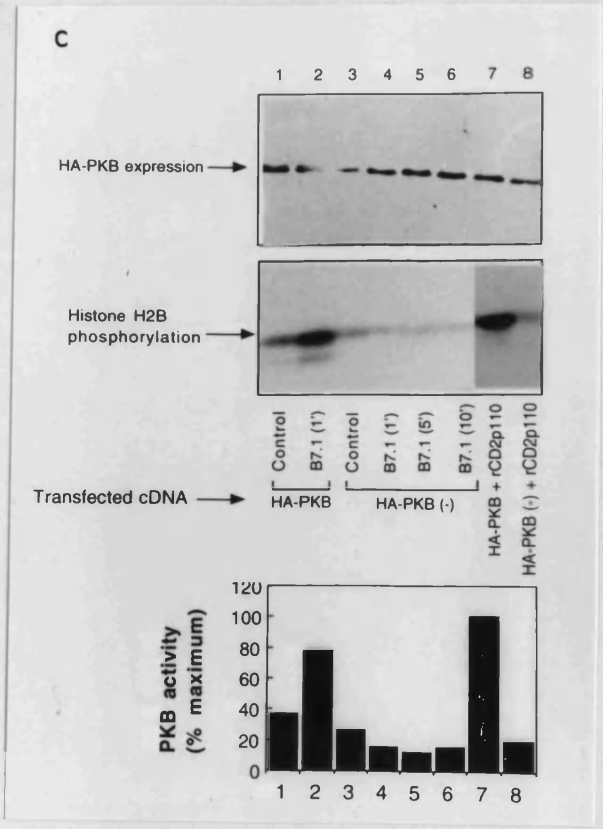


Figure 5.1.2 Activation of PKB in Jurkat cells following CD28 ligation. Jurkat cells were transiently transfected with HA-PKB (a, b and c) or kinase dead HA-PKB(-) (c). The Jurkat cells were left unstimulated (control/C) or stimulated with 1µg/ml anti-CD28 mAb 9.3 (a), $0.5x10^6$ CHO-B7.1$^+$ (a, b and c) or parental CHO cells (a) for the times indicated. (b) CHO-B7.1$^+$ cells were incubated for 5 min with 1µg/ml CTLA4-Ig fusion protein prior to cosedimentation and incubation with Jurkat cells which had been transfected with HA-PKB. Where indicated, the Jurkat cells were pretreated for 10 minutes with 100 nM wortmannin or 10 µM LY294002. After cell lysis and immunoprecipitation with anti-HA mAb, the immunoprecipitates were subjected to a PKB assay using histone H2B as a substrate. The reactions were stopped and proteins resolved by SDS-PAGE. The upper part of the gel was transferred to PVDF and immunoblotted with anti-PKBα polyclonal antibody (upper panel). The lower part of the gel was dried and histone H2B phosphorylation was detected by autoradiography at -80^{o}C (middle panel) followed by densitometric analysis (lower panel). Data are from a single representative experiment.

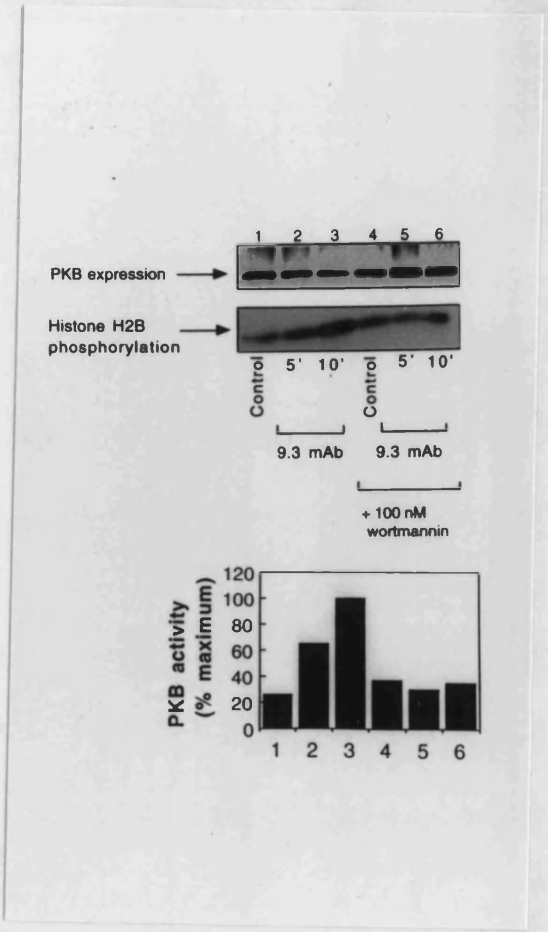


Figure 5.1.3 B7 ligation activates PKB in normal T lymphocytes. Normal T lymphocytes were purified by negative selection (as described section 2.2.3). The cells were either left unstimulated (control) or stimulated with 10 μg/ml anti-CD28 mAb 9.3 for the times indicated. Where indicated T lymphocytes were pretreated with 100 nM wortmannnin. After cell lysis and immunoprecipitation with anti-HA mAb, the immunoprecipitates were subjected to a PKB assay using histone H2B as a substrate. The reactions were stopped and proteins resolved by SDS-PAGE. The upper part of the gel was transferred to PVDF and immunoblotted with anti PKBα polyclonal antibody (upper panel). The lower part of the gel was dried and histone H2B phosphorylation was detected by autoradiography (middle panel) followed by densitometric analysis (lower panel). Data are from single representative experiment.

5.1.4 PKB co-immunoprecipitates an unidentified phosphoprotein.

In an attempt to generate PKB phosphorylated with ^{32}P for phosphopeptide mapping to verify that CD28 could also stimulate phosphorylation of ^{308}Thr, Jurkat cells were transiently transfected with HA-PKB or co-transfected with HA-PKB and the rCD2p110 chimaera and incubated for sixteen hours. The transiently transfected cells were then radiolabelled with [^{32}P] Pi before treatment with various stimuli, prior to lysis and immunoprecipitation of HA-PKB. A phosphorylated protein was observed to co-immunoprecipitate with the HA-PKB however, which migrated with an apparent molecular weight of approximately 180 kDa. Phosphorylation of this protein was induced by co-transfection of Jurkat cells with constitutively active PI 3-kinase, or treatment of cells with either anti-CD28 or anti-CD95 mAb (Figure 5.1.4). This unidentified phosphoprotein may represent a PKB interacting protein.

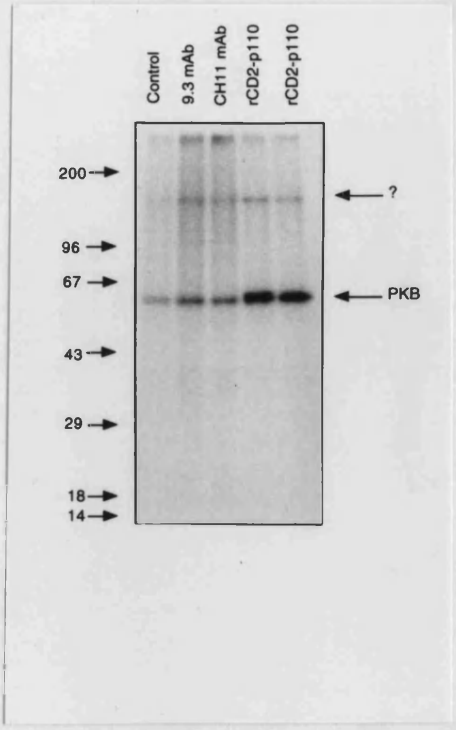


Figure 5.1.4 PKB co-immunoprecipitates with a phosphoprotein of approximately 180 kDa. Jurkat cells were transiently transfected with HA-PKB or co-transfected with HA-PKB and rCD2p110. The Jurkat cells were left unstimulated (control) or stimulated with 1μg/ml anti-CD28 mAb 9.3 or anti-CD95 mAb (CH11) for five minutes as described in the annotation. After cell lysis and immunoprecipitation with anti-HA mAb, the immunoprecipitated proteins were resolved by SDS-PAGE. The gel was dried and phosphorylated proteins visualised by autoradiography at -80°C. Data is from a single representative experiment.

5.2 CD28 mediates activation of p70 S6 kinase

P70 S6 kinase is a mitogen activated serine/threonine kinase that is indicated as a downstream effector of PI 3-kinase by a number of lines of evidence. i) PI 3-kinase is required for the phosphorylation of ^{252}T in the catalytic domain of p70 S6 inase [Weng *et al.* (1995)] and ii) activation of the enzyme by insulin or PDGF is sensitive to wortmannin [Chung *et al.* (1994)]. Furthermore, p70 S6 kinase has been demonstrated as necessary for G1 to S phase progression within the cell division cycle [Reinhard *et al.* (1994)], thus p70 S6 kinase would fit well as a downstream effector of CD28. Accordingly, experiments were performed to examine the effects of CD28 activation on p70 S6 kinase activity.

In this study the PI 3-kinase inhibitor wortmannin has been used to assess the role of PI 3-kinase signalling to couple CD28 to p70 S6 kinase. Jurkat cells were removed from culture, washed and aliquoted at 7.5x 10^6 cells/point prior to stimulation. After appropriate vehicle/drug stimulations, the Jurkat cells were lysed and cell lysates assayed for p70 S6 kinase activity by immunoblotting using a polyclonal antibody which recognises both the αI and αII isoforms of p70 S6 kinase. p70 S6 kinase activity was determined by the appearance of a slow mobility αII isoform on SDS-PAGE 7-17% acrylamide gradient gels. Initial attempts to demonstrate CD28 mediated activation of p70 S6 kinase were hampered by the high basal activity of the enzyme. Accordingly, cells were serum starved overnight, prior to stimulation. Serum starvation did not affect viability as assessed by trypan blue exclusion. Treatment of Jurkat cells with the PKC-activating phorbol ester, phorbol-12-myristate-13-acetate (50 ng/ml) resulted in the activation of p70 S6 kinase (Figure 5.2.1). Activation of p70 S6 kinase, following stimulation of Jurkat cells with 9.3 mAb (10 μg/ml) or PMA (50 ng/ml), was observed in the time range 1-30 minutes (Figure 5.2.1). This CD28-dependent activation of p70 S6 kinase was inhibited by a ten minute pretreatment with rapamycin (0.1-20 ng/ml), but was resistant to pretreatment with cyclosporin A (0.1-10 μg/ml) (Figure 5.2.2). Pretreatment of Jurkat cells with nanomolar concentrations of wortmannin, previously shown to inhibit CD28-induced accumulation of the lipid products of PI 3-kinase (section 3.1), resulted in inhibition of CD28 induced activation of p70 S6 kinase (Figure 5.2.3). Rapamycin, which potently inhibited CD28 mediated p70 S6 kinase activation, had no effect on the accumulation of PtdIns(3,4,5)P_3 (Figure 5.2.4), suggesting that the effects of rapamycin on CD28-stimulated p70 S6 kinase activation were not due to upstream inhibition of PI 3-kinase. These data indicate that activation of p70 S6 kinase is at least one downstream target for the PI 3-kinase signalling pathway following CD28 ligation on T cells.

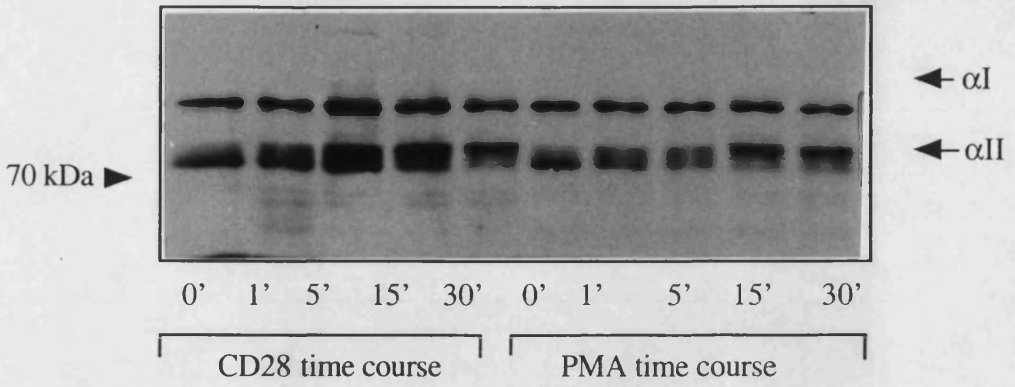


Figure 5.2.1 Activation of p70 S6 kinase by CD28 and PMA. Jurkat cells ($7.5x10^6$) were treated with 10 ng/ml PMA or 10 µg/ml CD28.2 (anti-CD28) mAb, as detailed in the legend. Cells were lysed in ice-cold NP40 lysis buffer and subjected to SDS-PAGE on a 7-17% acrylamide gradient gel. Separated proteins were transferred to PVDF membrane as described (section 2.2.10). Western blots were then analysed by immunoblotting with anti p70 S6 kinase antibody and activation state of the enzyme assessed by the appearance of electrophoretically retarded αII isoforms.

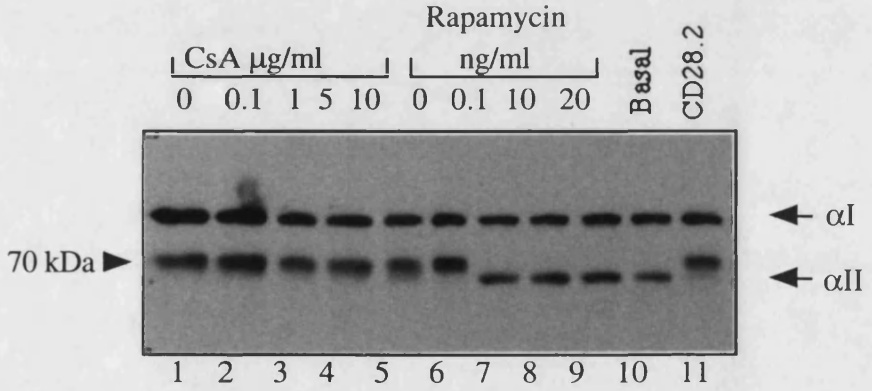


Figure 5.2.2 CD28 mediated activation of p70 S6 kinase is inhibited by rapamycin but not cyclosporin A. Serum starved Jurkat cells ($7.5x10^6$) were pre-incubated with cyclosporin A or rapamycin (as detailed in the annotation) for thirty minutes. Cells were then treated with CD28.2 (anti-CD28) mAb (except lane 10), for thirty minutes at 37°C. Cells were lysed in ice-cold NP40 lysis buffer and subjected to SDS-PAGE on a 7-17% acrylamide gradient gel. Separated proteins were transferred to PVDF membrane as described (section 2.2.10). Western blots were then analysed by immunoblotting with anti p70 S6 kinase antibody and activation state of the enzyme assessed by the appearance of electrophoretically retarded αII isoforms.

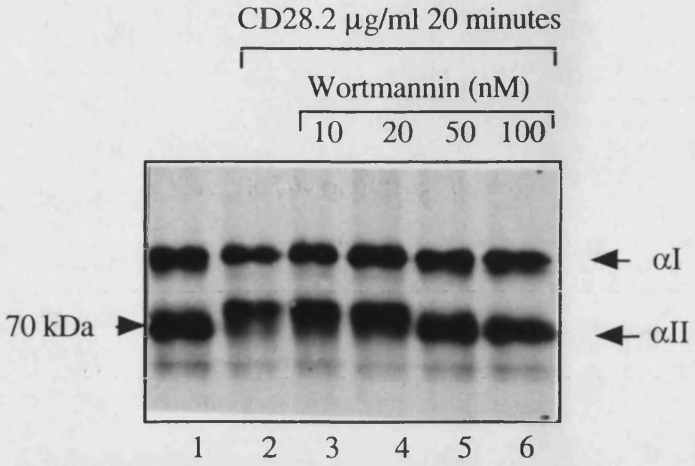


Figure 5.2.3 CD28 mediated activation p70 S6 kinase is inhibited by wortmannin. Serum starved Jurkat cells (7.5x 10^6/point) were pretreated with wortmannin (lanes 3 to 6, 100 nM, 10 minutes) before treatment with CD28.2 mAb for thirty minutes, (lanes 1 and 3 to 6) as detailed in the legend. Cells were lysed in ice-cold NP40 lysis buffer. Proteins were then resolved by SDS-PAGE on 7-17% acrylamide gradient gels and transferred to PVDF membrane, as described (section 2.2.10). Western blots were then immunoblotted with anti-p70 S6 kinase antibody and activation assessed by the appearance of electrophoretically retarded αII isoforms.

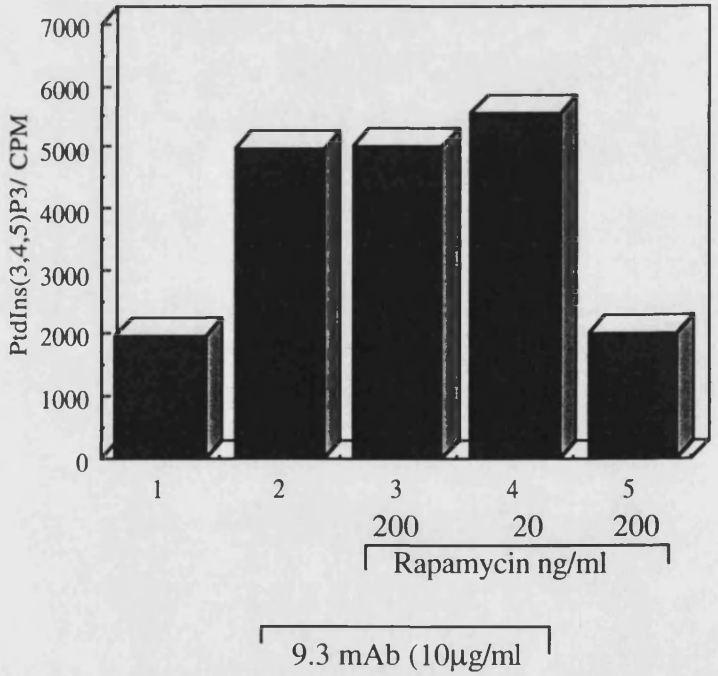


Figure 5.2.4 Rapamycin does not inhibit CD28 mediated PtdIns(3,4,5)P_3 accumulation in Jurkat cells. Jurkat cells were radiolabelled with ^{32}P, as described (section 2.2.11). Cells were then aliquoted (2x10^7/point) prior to stimulation with 9.3 mAb (10 µg/ml) for five minutes before chloroform extraction of cellular lipids. Lipids were then solubilised in water by deacylation and subjected HPLC analysis, as described (see section 2.2.14). Data are absolute counts from single representative experiment.

5.3 CD28 activates an SH2 containing inositol 5-phosphatase.

Since PtdIns(3,4,5)P_3 is the major lipid product of CD28-activated PI 3-kinase, it is important to understand the mechanisms by which levels of this signalling molecule are regulated. In this respect experiments were performed to assess the role of the D-5 phosphatase SHIP in CD28 signal transduction. This study has used immunoblotting techniques to show that SHIP is expressed in DC27.1 cells but not Jurkat (Figure 5.3.1). Thus, in subsequent experiments designed to investigate SHIP function, murine hybridoma DC27.1 cells, stably transfected with cDNA for human wild type CD28 [Pages *et al.* (1994)] have been used. The anti-SHIP antibody also cross-reacted with bands of approximately 100 kDa and 190 kDa (Figure 5.1.3) and these may represent alternative isoforms of SHIP. Previous studies have demonstrated SHIP to be heavily tyrosine phosphorylated in response to treatment with macrophage-colony stimulating factor (M-CSF), in 32D cells expressing Fms, a tyrosine kinase growth factor receptor, closely related to the platelet derived growth factor receptor (PDGF), which binds homodimeric M-CSF [Lioubin *et al.* (1996)]. Accordingly, it was investigated as to whether CD28 ligation induced tyrosine phosphorylation of SHIP in DC27.1 cells. Firstly, DC27.1 cells were stimulated with the anti-CD28 mAb 9.3, prior to immunoprecipitation of SHIP, and *in vitro* protein kinase assays were performed. CD28 ligation was found to induce rapid and sustained phosphorylation of a 145 kDa substrate (Figure 5.3.2), which is assumed to be SHIP. Phosphorylation of the 145 kDa substrate was accompanied by phosphorylation of further molecules of approximately 40 and 100 kDa, the identity of which are unknown (Figure 5.3.2). In an alternative strategy to demonstrate SHIP as tyrosine phosphorylated in response to CD28 ligation, DC27.1 cells were stimulated with 9.3 mAb and 2C11 mAb (which ligates murine CD3), and SHIP immunoprecipitated. Phosphorylated proteins were then detected by immunoblot analysis with 4G10 mAb. Ligation of human CD28 and murine CD3 were both found to induce tyrosine phosphorylation of SHIP within 1 minute which was sustained for at least 10 minutes, in DC27.1 cells (Figure 5.3.3 a and c). Furthermore, these induced phosphorylation events were demonstrated as antigen-specific since treatment of cells with rabbit anti-mouse immunoglobulins and UCHT1 did not induce detectable tyrosine phosphorylation of SHIP (Figure 5.3.3 a and c). Blots were stripped and reprobed with anti-SHIP Ab to demonstrate equal loading of proteins (Figure 5.3.3.b and d) and verify that the position of the tyrosine phosphoprotein correlated with the position of SHIP on SDS-PAGE.

Having demonstrated the tyrosine phosphorylation of SHIP in response to CD28 and CD3 ligation, further experiments were performed to determine if tyrosine

phosphorylation of the molecule correlated with activation of its catalytic function. Accordingly, SHIP activity was assessed by measuring the ability of the SHIP immunoprecipitates to hydrolyse [^{3}H]-Ins(1,3,4,5)P_4. Hydrolysis of [^{3}H]-Ins(1,3,4,5)P_4 by SHIP should result in the formation of Ins(1,3,4)P_3, and it is possible to separate and measure these water soluble inositol polyphosphates using HPLC, and on-line β-scintilation counting. Figure 5.3.4 demonstrates that SHIP immunoprecipitates, derived from unstimulated DC27.1 cells, display a basal ability to degrade [^{3}H]-Ins(1,3,4,5)P_4. Treatment of cells with 9.3 mAb (10 μg/ml) for 1 to 10 minutes however, results in an increased ability to hydrolyse the substrate as characterised by the decrease in Ins(1,3,4,5)P_4 and formation of Ins(1,3,4)P_3. Similarly, ligation of the murine T cell antigen receptor with 2C11 mAb resulted in an increased hydrolysis of the Ins(1,3,4,5)P_4 substrate. These experiments demonstrate that ligation of CD28 or the CD3, results in increased activity of the newly identified SH2 containing inositol polyphosphate 5-phosphatase in murine hybridoma cells.

Since ligation of CD28 clearly induces tyrosine phosphorylation and activation of SHIP, CD28 immunoprecipitates from murine DC27.1 cells were immunoblotted with anti-SHIP antibody in an attempt to demonstrate a direct association between the two molecules. Under conditions where it was possible to detect p85 associated with CD28 however, no SHIP could be detected in CD28 immunoprecipitates (Figure 5.3.5). This suggests that SHIP does not directly interact with CD28.

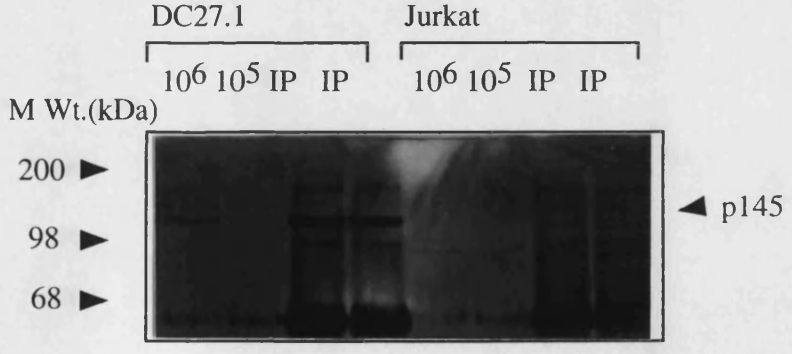


Figure 5.3.1 SHIP is expressed in murine DC27.1 cells but not Jurkat. Cellular lysates (cell density shown in annotation) and SHIP immunoprecipitates from (10^7) DC27.1 or Jurkat cells (10^7) were resolved by SDS-PAGE on 10% homogeneous acrylamide gels. Separated proteins were transferred to nitrocellulose membranes and immunoblotted for the presence of SHIP.

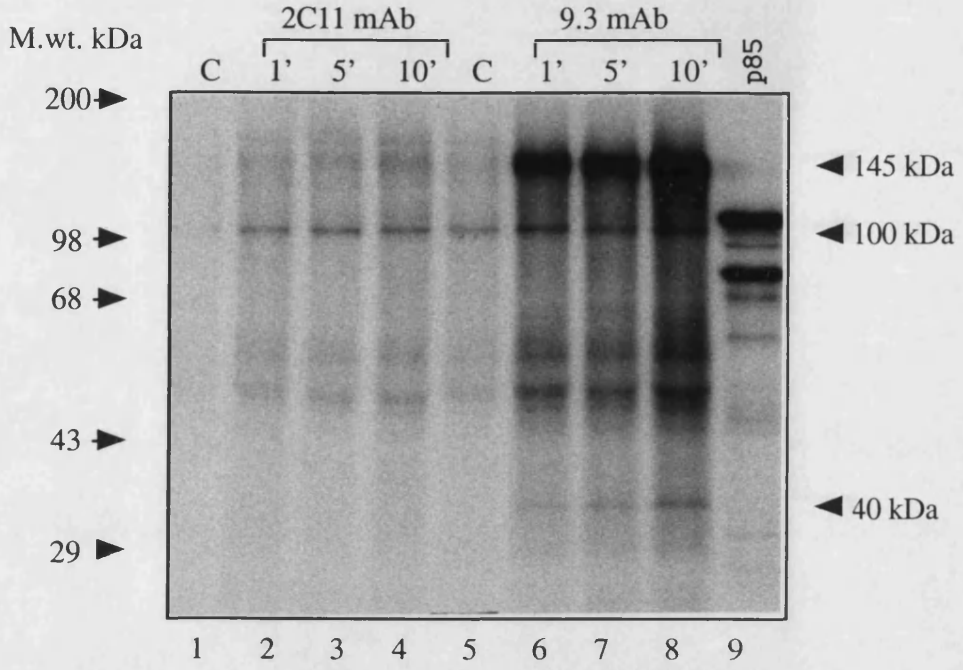


Figure 5.3.2 ***In vitro*** **kinase assays on SHIP immunoprecipitates show phosphorylation of 145 kDa, 100 kDa and 40 kDa substrates.** SHIP (lanes 1 to 8) and p85 positive control (lane 9) immunoprecipitates were prepared from DC27.1 cells expressing wild type human CD28, that had been stimulated with 10 μg/ml 2C11 mAb (lanes 2 to 4) or 10 μg/ml 9.3 mAb (lanes 6 to 8). Immunoprecipitates were washed and *in vitro* protein kinase assays carried out as described (section 2.2.17). Proteins were subjected to SDS-PAGE on a 7-17% acrylamide gradient gel which was subsequently fixed and dried. Phosphorylated proteins were visualised by autoradiography at -80°C. Data are from single representative experiment.

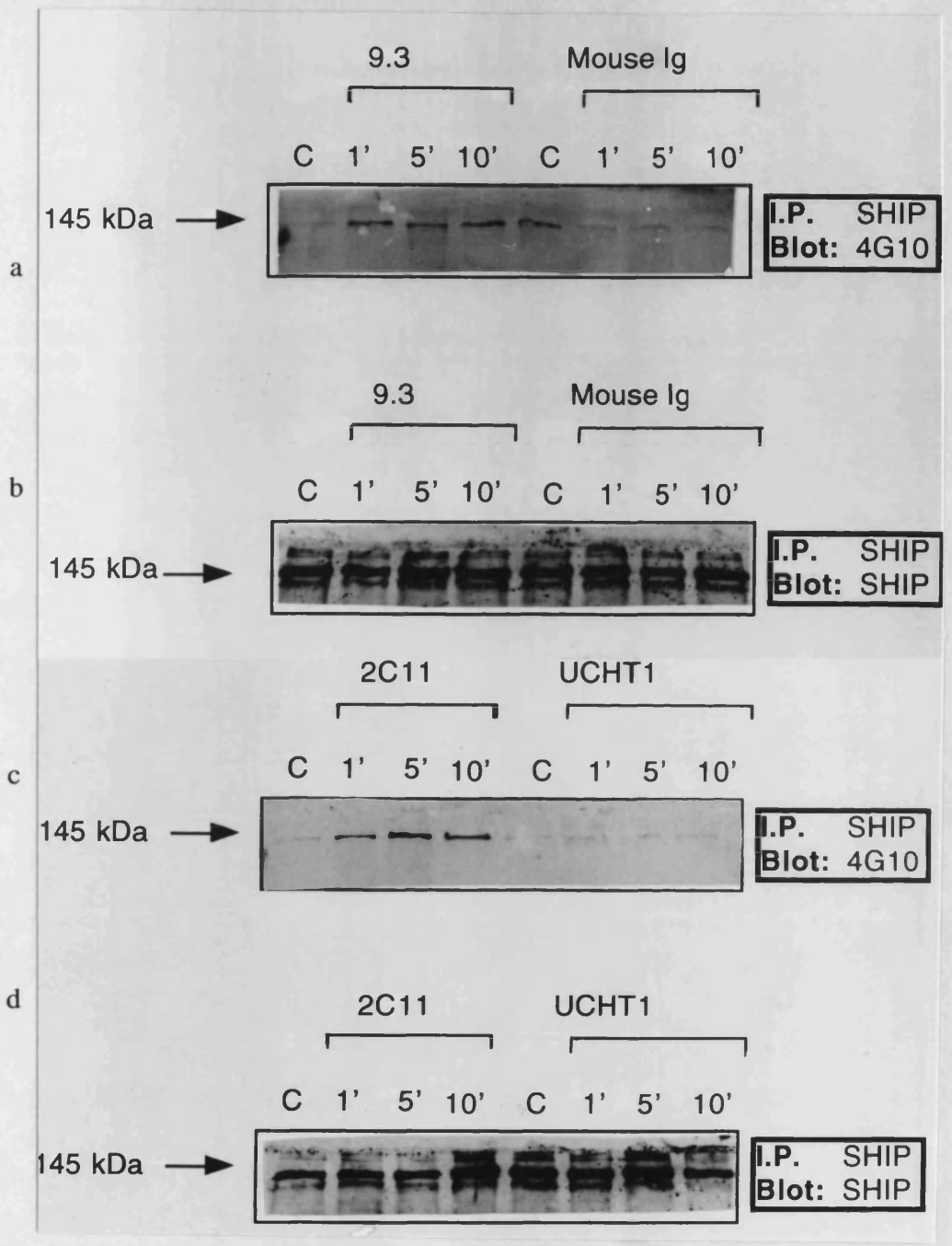


Figure 5.3.3 SHIP is tyrosine phosphorylated in response to CD28 and CD3 ligation. SHIP was immunoprecipitated from 10^7 wild type human CD28 expressing DC27.1 cells which had previously been stimulated according to the annotation. 9.3 mAb ligates CD28, RaM (rabbit anti-mouse) was used as a general non-specific Ig to test specificity of the 2C11 and 9.3 reponses (panels a and b), 2C11 mAb ligates the mouse T cell antigen receptor and UCHT1 mAb ligates the human T cell antigen receptor (panels c and d). Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was then carried out to identify tyrosine phosphorylated proteins using the 4G10 mAb (panels a and c). The western blots were then stripped and reprobed with anti-SHIP antibody (panels b and d) to demonstrate equal loading of the proteins. Data are fdrom single representative experiment.

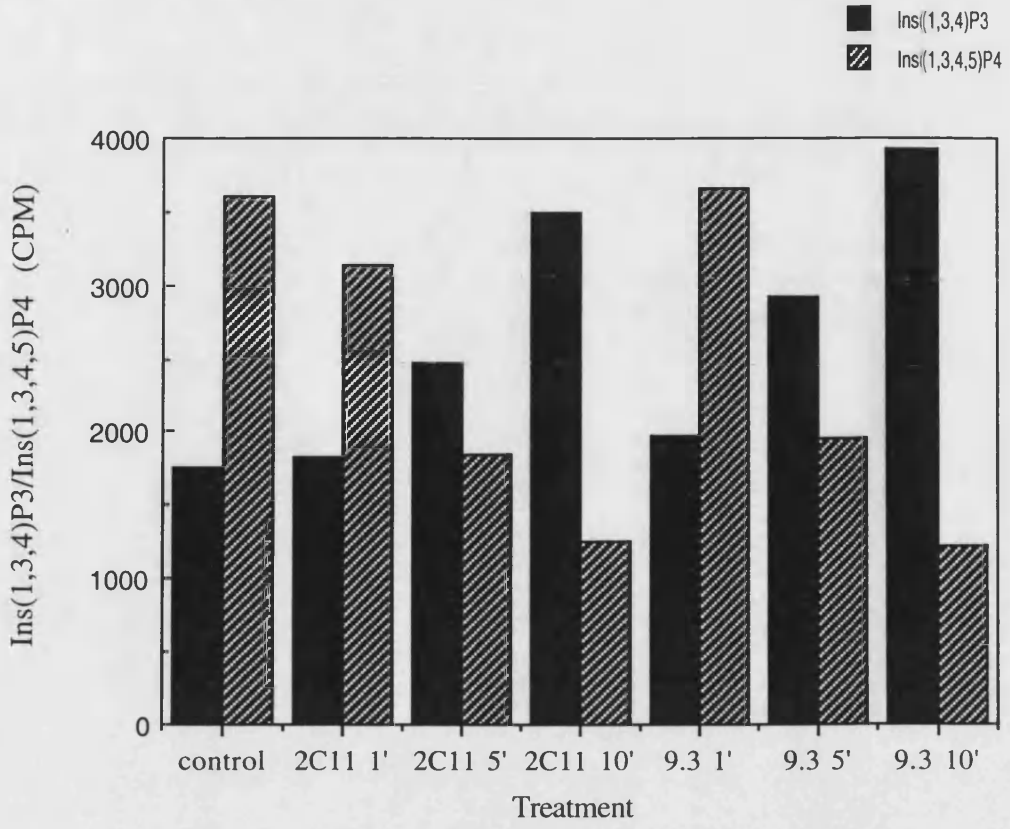


Figure 5.3.4 Effect of CD28 ligation on SHIP activity. SHIP was immunoprecipitated from 2C11 mAb (CD3) or 9.3 mAb treated murine DC27.1 cells. SHIP activity was then assessed by the ability of the immunoprecipitates to hydrolyse [^{3}H]-Ins(1,3,4,5)P4 substrate (see section 2.2.20) . Substrate and products from the 5-phosphatase reaction were then quantitated by HPLC. Data are absolute counts from single representative experiment.

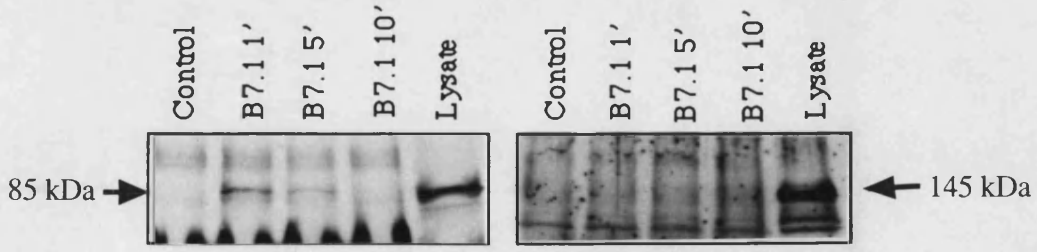


Figure 5.3.5 SHIP does not co-associate with CD28. Murine DC27.1 cells, stably transfected to express human CD28 (10^7 cells/point), were co-sedimented with CHO-B7.1$^+$ cells (0.5x 10^6 cells/point). After the times indicated, cells were lysed in ice cold NP40 lysis buffer and CD28 immunoprecipitated with 9.3 mAb. Proteins were resolved in duplicate by SDS-PAGE and transferred to PVDF membrane before immunoblot analysis with anti-p85 mAb (left hand panel) or anti-SHIP antibody (right hand panel).

5.4.1 CD28 activation of MAP kinases.

The MAP kinase family of protein represents a major intracellular signalling system with three pathways delineated in mammalian cells, controlling the activation of the ERKs, JNKs and p38 [for review see Kyriakis and Avruch (1998)]. Evidence exists for the involvement of PI 3-kinase in either or both of signalling cascades that control ERK or JNK, for example fos transcription, which is regulated by ERK or JNK activity, can be activated by a constitutively active p110 mutant [Hu *et al.* (1995)]. Since this is a ras dependent effect, it implies that PI 3-kinase may modulate the $p21^{ras}$ pathway and thus the eventual activation of the ERKs. Interestingly JNK has been reported as synergistically activated by CD28 and TCR ligation in Jurkat cells. [Su *et al.* (1994)]. Additionally, CD28 regulatory input into the MAP kinases has been reported via p21-CDC42/Rac which lie upstream of PAK. This has been suggested as a point of integration for CD3 and CD28 generated signals [Kaga *et al.* (1998)]. Given that CD28 may be able to regulate MAP kinases such as JNK, experiments were performed to examine the effect of CD28 ligation on JNK activity and the effect of MAP kinase inhibitors on proliferative signalling.

5.4.2 CD28 activates JNK.

In this study c-jun N-terminal kinase (JNK) has been immunoprecipitated using a recombinant protein corresponding to residues 1-135 of c-jun. JNK activity was then assessed by the ability of the immunoprecipitated protein to phosphorylate the co-asociated c-jun N-terminal protein in an *in vitro* kinase assay [Hibi *et al.* (1993)]. To demonstrate the effectiveness of the assay, cells were stimulated with UV light since JNK is activated by environmental stress, or PMA (10 ng/ml) in combination with ionomycin (1 μg/ml). Exposure to UV light was demonstrated to result in the activation of JNK (Figure 5.4.2a), as did PMA (10 ng/ml) in combination with ionomycin (1 μg/ml) (Figure 5.4.2a). Alone, PMA or ionomycin induced little or no JNK activation (Figure 5.4.2b). Similarly CD28 mAb alone had little or no activating effect on JNK activity, whilst in combination, PMA (10 ng/ml) and ionomycin (1 mg/ml) induced JNK activation which was potentiated by anti-CD28 mAb CD28.2 (10 μg/ml) (Figure 5.4.2b). Pre-incubation of cells with wortmannin was found to inhibit CD28 potentiation of JNK activity whilst no affect on PMA/ionomycin stimulated JNK activity was detected (Figure 5.4.2c).

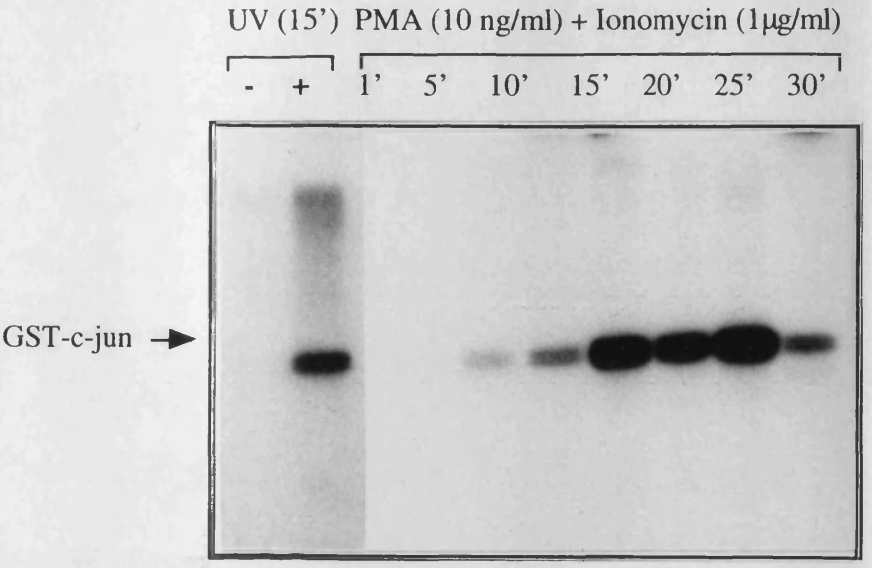


Figure 5.4.2a Activation of JNK by UV and PMA/ionomycin. Jurkat cells (5x 10^6/point) were stimulated, as detailed in the annotation, and lysed as described (see section 2.2.19). JNK was immunoprecipitated in complex with GST-c-jun, JNK activity was subsequently assessed by the ability of JNK to phosphorylate the GST c-jun fusion protein in an *in vitro* kinase assay (see section 2.2.19). Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography at -80°C.

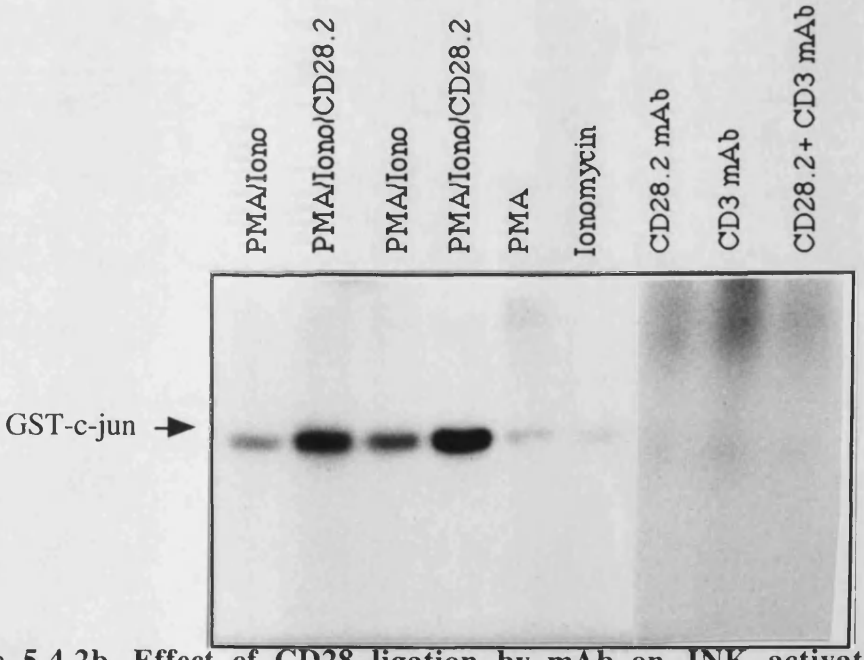


Figure 5.4.2b Effect of CD28 ligation by mAb on JNK activation. Jurkat cells (5x 10^6/point) were stimulated, as detailed in the annotation, and lysed as described (see section 2.2.19). JNK was immunoprecipitated in complex with GST-c-jun, JNK activity was subsequently assessed by the ability of JNK to phosphorylate the GST c-jun fusion protein in an *in vitro* kinase assay (see section 2.2.19). Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography at -80°C.

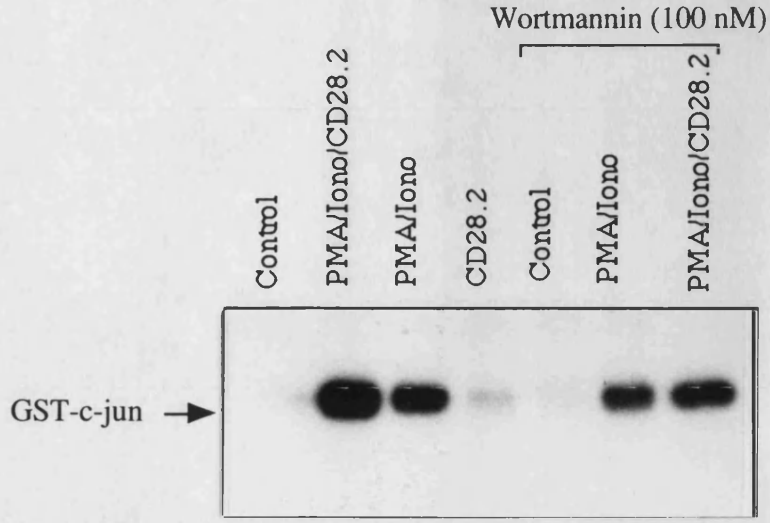


Figure 5.4.2.c Effect of wortmannin on JNK activation. Jurkat cells (5x 10^6/point) were stimulated, as detailed in the annotation, and lysed as described (see section 2.2.19). JNK was immunoprecipitated in complex with GST-c-jun, JNK activity was subsequently assessed by the ability of JNK to phosphorylate the GST c-jun fusion protein in an *in vitro* kinase assay (see section 2.2.19). Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography at -80°C.

5.4.3 The effect of MAP kinase inhibitors on T cell proliferation.

The availability of a selective and potent pyrinidyl imidazole inhibitor of p38 MAP kinase in SB203580 has enabled the identification of functional activities mediated by p38. Since CD28 may be implicated as regulating at least some MAP kinase activity (Figure 5.42c), I have used SB203580 to assess the importance of p38 in CD28 dependent T cell proliferation. The [^{3}H]-thymidine incorporation induced by anti-CD3 mAb and B7.1 treatment of cells was inhibited (>70%) in a concentration dependent manner (Figure 5.4.3.a) by one hour pretreatment of with SB203580 (IC_{50}=10± 2.5 μM, n=4). The [^{3}H]-thymidine incorporation induced by PMA and CD28 was also inhibited by SB203580 at similar concentrations. In contrast SB203580 was much less effective at inhibiting [^{3}H]-thymidine incorporation induced by the combination of 5 ng/ml PMA and 1 μM ionomycin (Figure 5.4.3b). Activation of the extracellular regulated kinases (ERKs) is mediated by MEK, which can be selectively inhibited by PD98059 [Dudley *et al.* (1995)]. Experiments were performed to determine the effect of PD98059 on CD28 mediated T cell proliferation. An hour pretreatment with PD98059 (0.1 to 50 μM) had no effect on [^{3}H]-thymidine incorporation induced by PMA and B7.1 treatment of cells (Figure 5.4.4a) however, [^{3}H]-thymidine incorporation induced by CD3 and CD28 (Figure 5.4.4a) was slightly inhibited (<50%), albeit at a lesser extent than the inhibition observed with similar concentrations of SB203580. PD98059 did not inhibit [^{3}H]-

thymidine incorporation in response to 5 ng/ml PMA and ionomycin (Figure 5.4.4b).

The effect of SB203580 on CD28-dependent IL-2 production was assessed by taking supernatants from human T cell cultures 24 hours after treatment with UCHT1 and CHO-B7.1$^+$ cells, in the absence or presence of SB203580. These supernatants were assayed for IL-2 production by a bioassay using the IL-2 dependent cell line CTLL [Rayter *et al.* (1992)]. This revealed that SB203580 inhibited IL-2-production from the T cells stimulated with anti-CD3 Ab and B7.1$^+$ (Figure 5.4.5a). The IC_{50} for inhibition of IL-2 production by SB203580 was 3± 1.4 μM, n=4, and was similar to that required for inhibition of [^{3}H]-thymidine incorporation (Figure 5.4.5b).

These data may indicate a role for p38 in CD28 driven IL-2 production. However, the SB203580 may be carried over in the supernatant assayed for IL-2, and may therefore inhibit IL-2 driven biochemical pathways, thus the observed inhibition of IL-2 production may be an artifact. Moreover a similar inhibition of IL-2 driven T cell proliferation may also explain the observed inhibition of CD28-dependent T cell proliferation. To investigate this possibility, human T lymphoblasts were stimulated with IL-2 (20 ng/ml) in the presence or absence of both SB203580 and PD98059 (Figure 5.4.6a+b). SB203580 was found to inhibit IL-2 driven proliferation of T lymphoblasts at 1-30 μM. In contrast 50 μM PD98059 had no effect on IL-2 stimulated T lymphoblast proliferation (Figure 5.4.6a+b). The IC_{50} for inhibition by SB203580 of sub-optimal IL-2 stimulated T lymphoblast proliferation was 5± 2.5 μM, n=4 (Figure 5.4.6b), and therefore similar to the IC_{50} values calculated for inhibition of anti-CD3 Ab and B7.1 driven IL-2 production and [^{3}H]-thymidine incorporation.

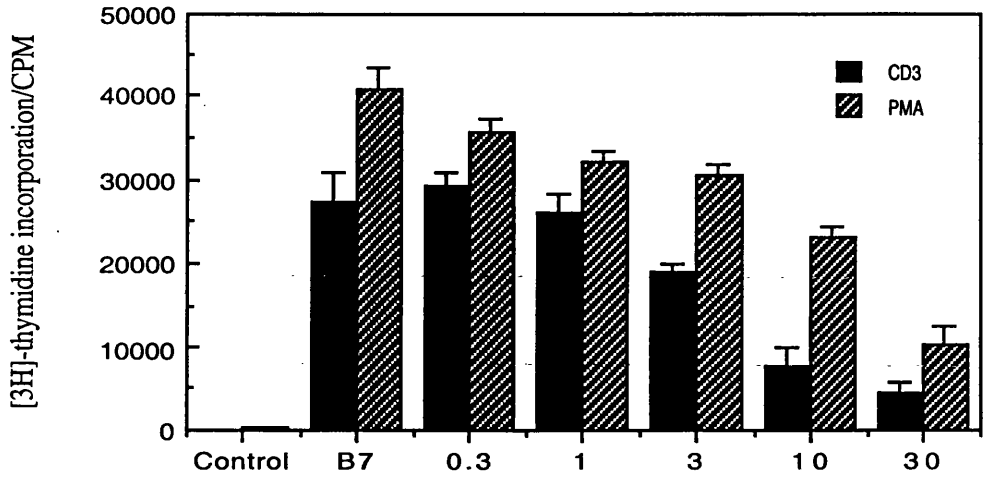


Figure 5.4.3 a: Effect of SB203580 on B7/CD3 or B7/PMA driven T cell proliferation. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were preincubated with SB203580 (at the concentrations indicated in the annotation) for one hour before treatment with CHO-B7.1$^+$ cells ($2x10^4$ cells/point) in combination with either anti-CD3 mAb UCHT1 (1 μg/ml) or PMA 5 ng/ml. Cells were pulsed with [^{3}H]-thymidine as described (section 2.2) before being harvested and assayed for [^{3}H]-thymidine incorporation, as described (see section 2.2.21). Data are mean of quintuplicate replicates from a single representative experiment.

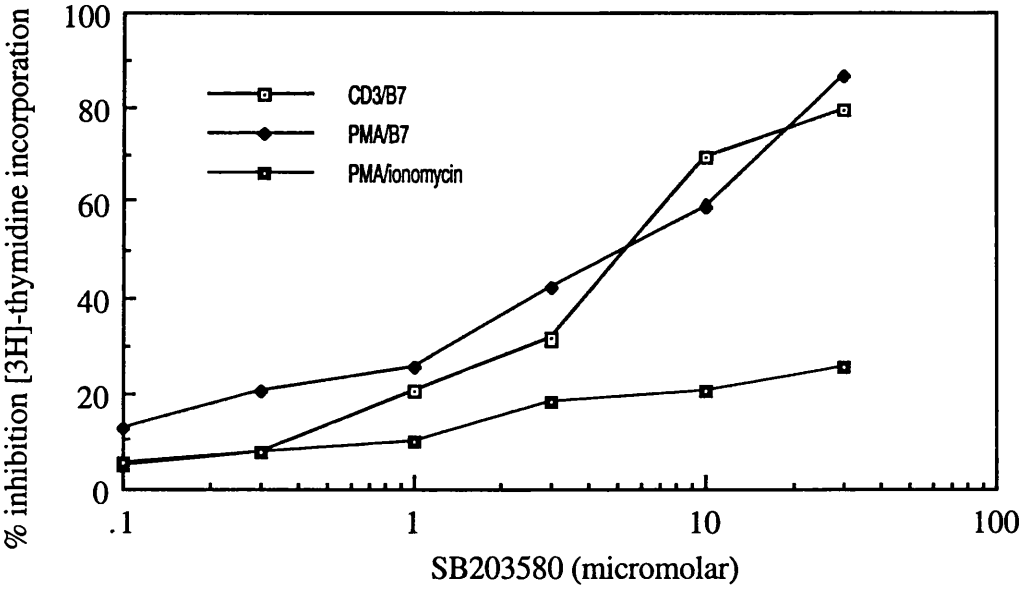


Figure 5.4.3 b: Inhibition of T cell proliferation by SB203580. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were preincubated with SB203580 (at the concentrations indicated in the annotation) for one hour before treatment with stimuli as indicated. CHO-B7.1$^+$ cells were included at $2x10^4$ cells/point, anti-CD3 mAb UCHT1 at 1 μg/ml or PMA 5 ng/ml and ionomycin at 1 μM. [^{3}H]-thymidine incorporation assays were performed, as described (see section 2.2.21). Data expressed as percentage inhibition of proliferation observed in control samples.

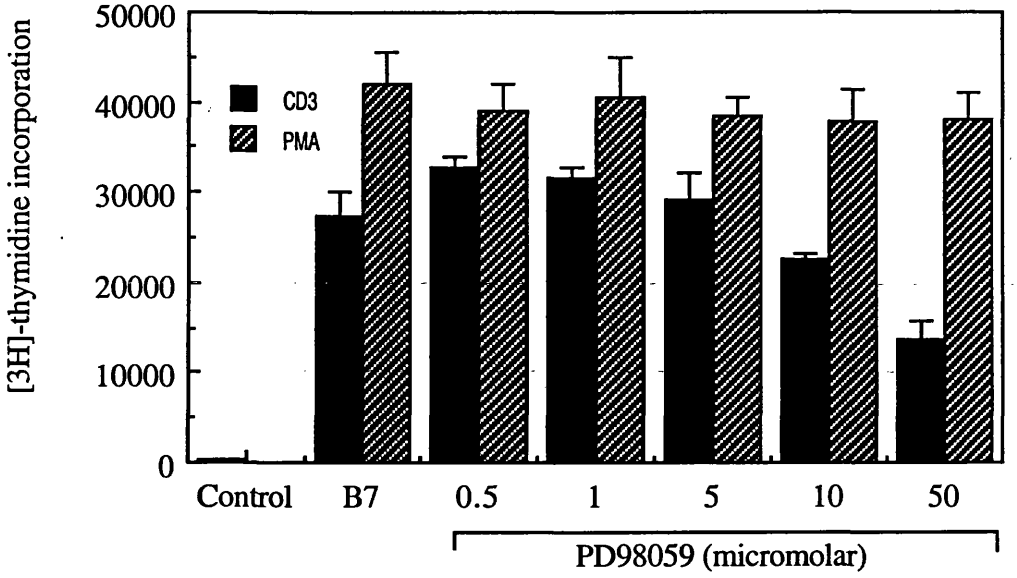


Figure 5.4.4 a: Effect of PD98059 on CD3/B7 or PMA/B7 driven T cell proliferation. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were pre-incubated with PD98059 (at the concentrations indicated in the annotation) for one hour before treatment with CHO-B7.1$^+$ cells ($2x10^4$ cells/point) in combination with either anti-CD3 mAb UCHT1 (1 μg/ml) or PMA 5 ng/ml. Cells were harvested and assayed for [^{3}H]-thymidine incorporation after 72 hours incubation, as described (see section 2.2.21). Data are mean and SEM of quintuplicate replicates from a single representative experiment.

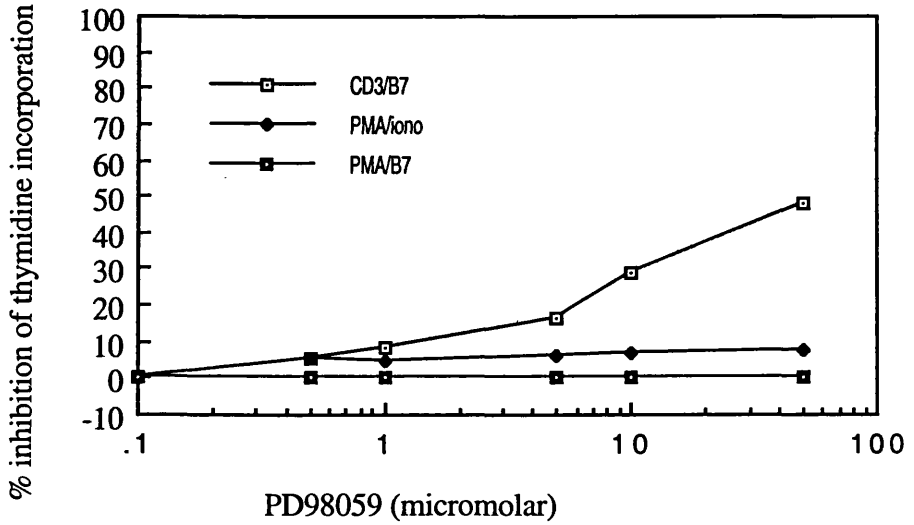


Figure 5.4.4 b: Inhibition of T cell proliferation by PD98059. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were preincubated with PD98059 (at the concentrations indicated in the annotation) for one hour before treatment with combinations of CHO-B7.1$^+$ cells ($2x10^4$ /point), anti-CD3 mAb UCHT1 (1 μg/ml), PMA 5 ng/ml or ionomycin (1 μM). Cells were harvested and assayed for [^{3}H]-thymidine incorporation after 72 hours incubation, as described (section 2.2.21). Proliferation driven by each treatment in the absence of inhibitors +/- S.E.M was CD3/B7= 27249± 3882 CPM, PMA/B7= 40883± 2380 CPM and PMA/Ionomycin= 18255± 3561 CPM. Data are mean of quintuplicate replicates from a single representative experiment.

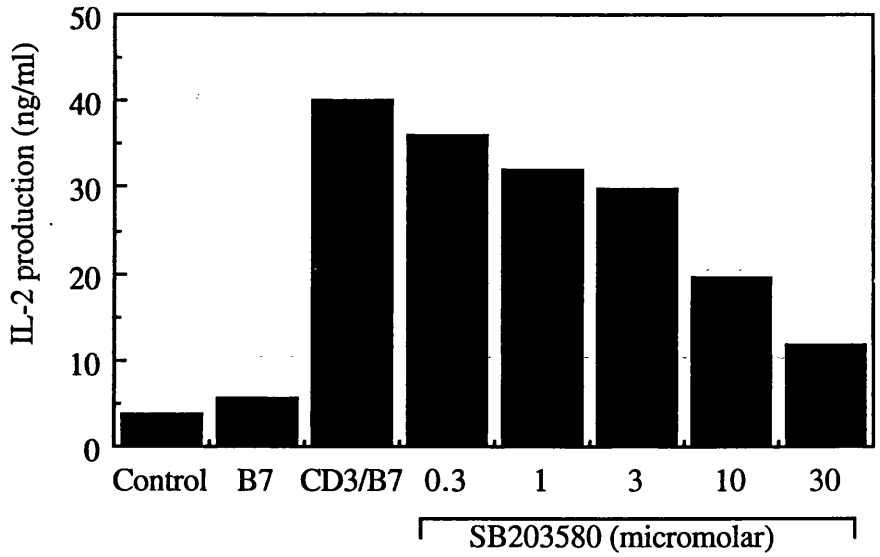


Figure 5.4.5 a: Effect of SB 203580 on CD3/B7 driven IL-2 production. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were treated with anti-CD3 mAb UCHT1 (1 μg/ml) and CHO-B7.1$^+$ ($2x10^4$ /point) in the presence of SB203580 at the concentrations indicated. Supernatants from the cell cultures were harvested after 24 hours and IL-2 production detected by a bioassay technique using the IL-2 dependent cell-line CTLL as described (section 2.2.22).

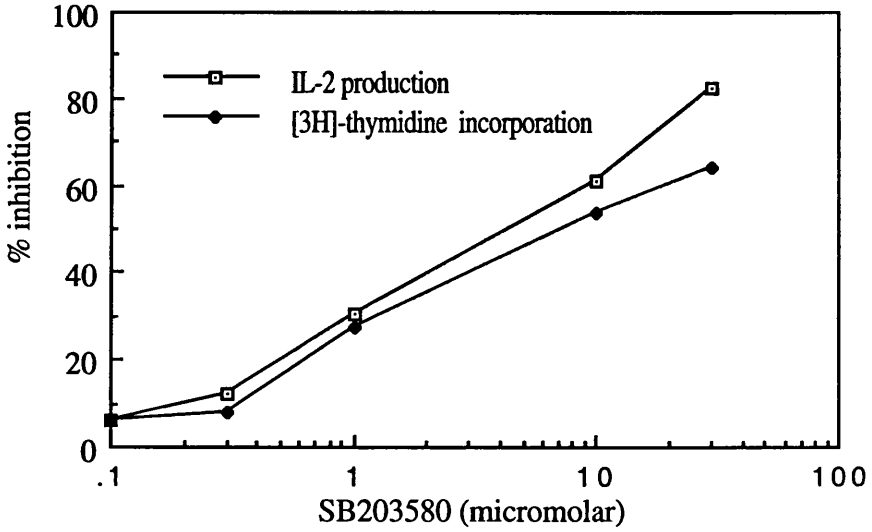


Figure 5.4.5 b: Effect of SB203580 on CD3/B7 driven T cell activation. Peripheral blood T cells ($5x10^4$ cells/point) derived from normal healthy donors were incubated for one hour in the presence of SB203580, at the concentrations indicated, prior to activation with anti-CD3 mAb UCHT1 (1 μg/ml) and CHO-B7.1$^+$ ($2x10^4$ /point) in two parallel experiments. Supernatants were harvested, from one plate, at 24 hours and assayed for IL-2 production (section 2.2.22). From the second plate [^{3}H]-thymidine incorporation was determined after 72 hours (section 2.2.21). Data are mean of quintuplicate replicates from single representative experiment.

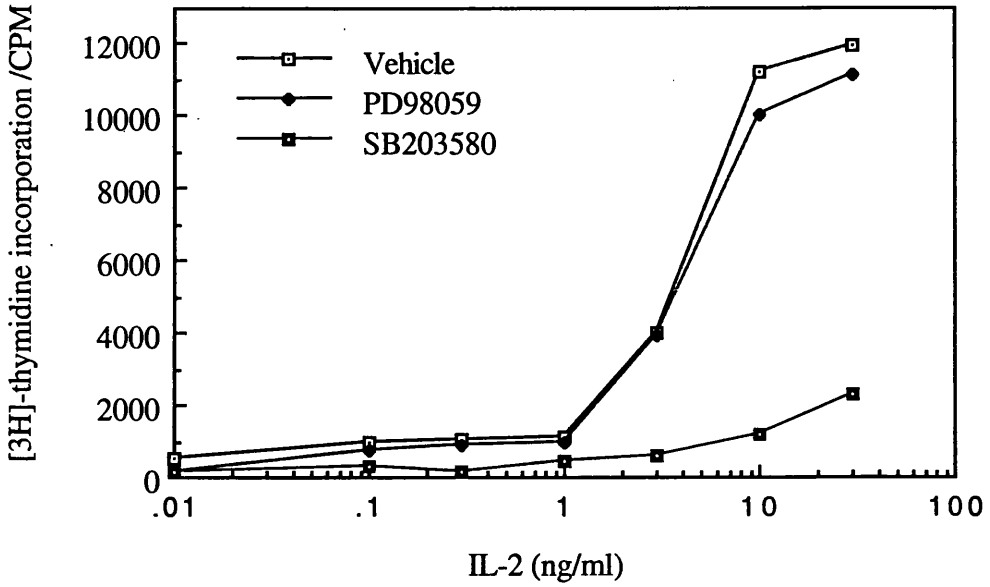


Figure 5.4.6 a Effect of SB203580 and PD98059 on IL-2 driven proliferation of T cell lymphoblasts. T cell lymphoblasts prepared as described (section 2.2.2) were preincubated with PD98059 (50 μM), SB203580 (10 μM) or untreated (vehicle), for one hour before incubation with IL-2 at the concentrations indicated. Cells were harvested and assayed for [^{3}H]-thymidine incorporation after 72 hours incubation, as described (see section 2.2.21). Data are mean of quintuplicate replicates from a single representative experiment.

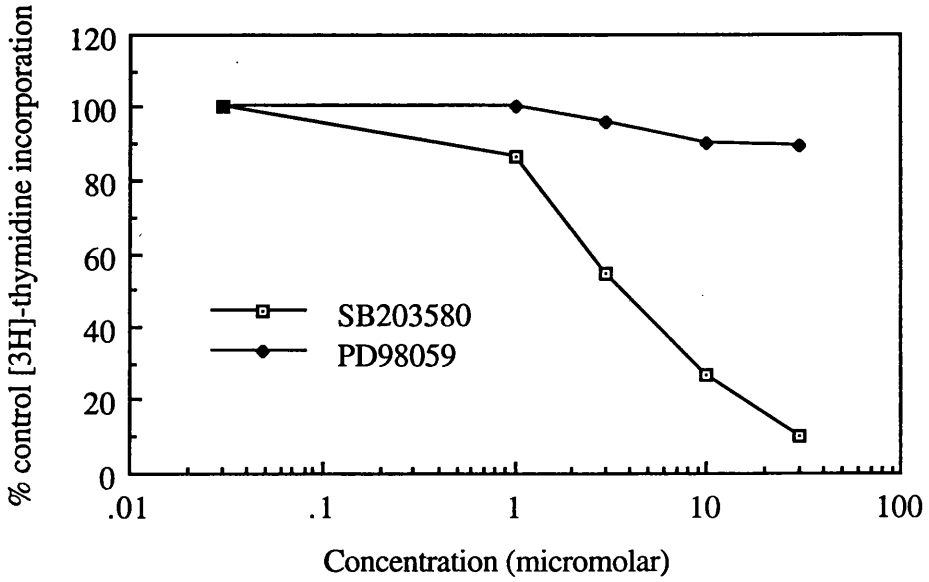


Figure 5.4.6 b: Effect of SB203580 and PD98059 on IL-2-stimulated [^{3}H]-thymidine incorporation in T lymphoblasts. T cell lymphoblasts prepared as described, were preincubated with PD98059 or SB203580 (at the concentrations indicated) for one hour before incubation with IL-2 (3 ng/ml). Cells were harvested and assayed for [^{3}H]-thymidine incorporation after 72 hours incubation, as described (see section 2.2.21). [^{3}H]-thymidine incorporation in unstimulated cells was 499± 69 CPM and IL-2 (3 ng/ml) stimulated cells was 4031± 579 CPM. Data are mean of quintuplicate replicates from a single representative experiment.

5.4.4 Effect of SB203580 on CD28 mediated activation of PI 3-kinase.

The observed effects of SB203580 on CD28-driven [^{3}H]-thymidine incorporation may be explained by non-specific inhibition of PI 3-kinase, inhibition of which is known to abrogate CD28-stimulated proliferation. To examine this possibility radiolabelled Jurkat cells were incubated with the MAP kinase inhibitor SB203580 (0 to 30 μM) prior to co-sedimentation with CHO-B7.1$^+$ cells and extraction and quantitation of the radiolabelled phospholipids. Treatment of Jurkat cells with SB203580 (0 to 30 μM) was found to be without effect on the CD28-mediated accumulation of PtdIns(3,4,5)*P*$_3$, (Figure 5.4.7). These data indicate that the inhibitory effect of the p38 MAP kinase inhibitor SB203580 on CD28-dependent T cell proliferation (Figure 5.2.3), is achieved independently of an effect upon PI 3-kinase.

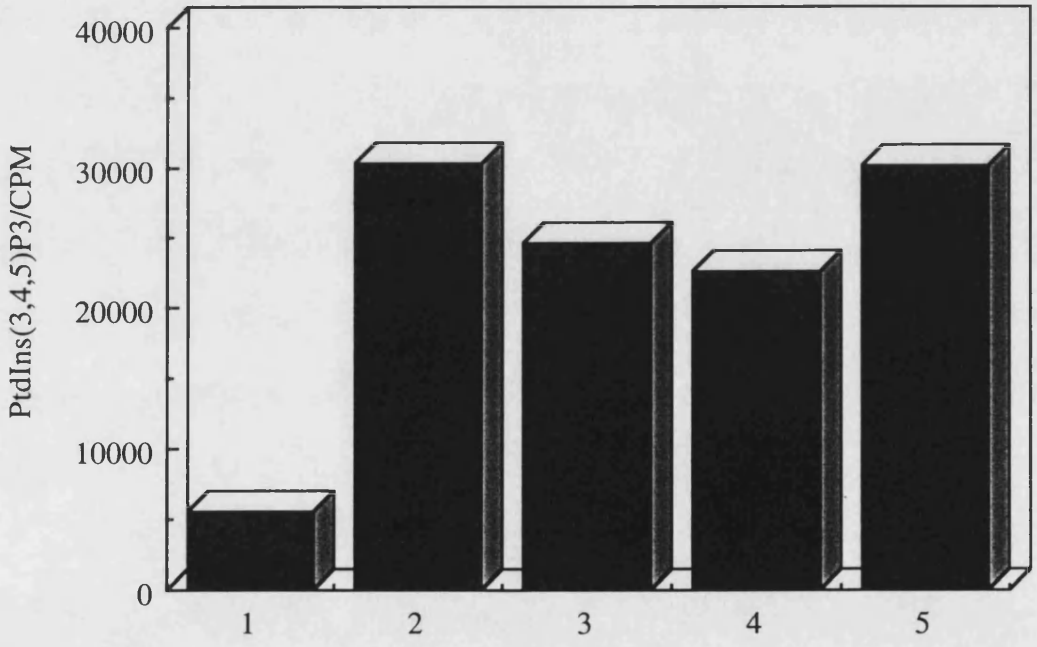


Figure 5.4.7 p38 MAP kinase inhibitor SB203580 does not affect PI 3-kinase activation. Jurkat cells were radiolabelled with ^{32}P, as described (see section 2.2.11). Cells were then aliquoted (2x10^7/point) and preincubated with SB203580 (lanes 3 to 5) at 0.3 μM (3), 3 μM (4) and 30 μM (5) for one hour. Cells were then cosedimented with CHO-B7.1$^+$ (10^7/point) for five minutes (lanes 2 to 5) before chloroform extraction of cellular lipids. Lipids were then solubilised in water by deacylation and subjected HPLC analysis, as described (see section 2.2.14). Data are from single representative experiment.

Section 5.5 Summary.

1) Protein kinase B has been confirmed as a downstream target for PI 3-kinase and demonstrated as a downstream target of CD28 in Jurkat cells and purified primary T cells by the use of *in vitro* kinase assays. Transiently expressed HA-PKB has also been demonstrated to co-immunoprecipitate from Jurkat cells with an unidentified phosphoprotein of approximately 180 kDa.

2) CD28 has been demonstrated to activate p70 S6 kinase in a PI 3-kinase dependent and rapamycin-sensitive manner. Since rapamycin had no effect on PI 3-kinase activation, rapamycin must exert its effect either downstream or on a parallel signalling pathway to PI 3-kinase.

3) CD28 ligation has been shown to induce tyrosine phosphorylation of SHIP and increase 5'-phosphatase activity associated with SHIP immunoprecipitates from murine DC27.1 cells. No direct association between SHIP and CD28 was detected however.

4) Whilst the specific p38 inhibitor SB203580, abrogates PMA/B7 and CD3/B7 driven T cell proliferation, it also inhibits IL-2 driven proliferation of T lymphoblasts. Hence, the inhibitory actions of SB203580 on CD28-dependent T cell proliferation might be explained by inhibition of IL-2 rather than CD28-stimulated proliferation.

5) CD28 has been demonstrated to potentiate JNK activation induced by PMA in combination with ionomycin, in a wortmannin sensitive manner.

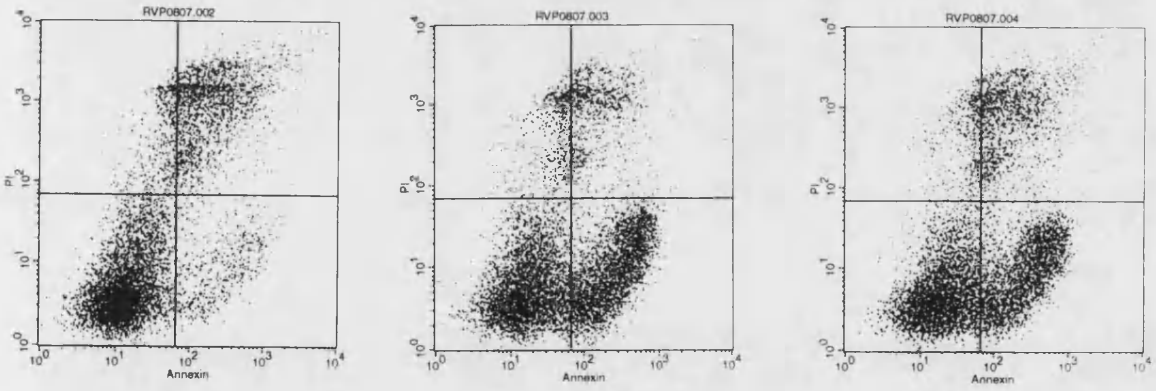
SECTION SIX

Activation of T cell costimulatory targets following CD95 ligation

6.1 CD95 mediates killing of Jurkat cells.

CD95 is a member of the TNFR/GFR superfamily which signal for a broad spectrum of functional outcomes including thymocyte proliferation (TNFR2) [Tartaglia *et al.* (1991)] and immunoglobulin class switching (CD40) [Kwabe *et al.* (1994)]. CD95 is widely accepted to trigger an apoptotic pathway when ligated [Itoh *et al.* (1991)], however there is evidence to support a positive, possibly costimulatory role for CD95-mediated signals. For example anti-CD95 Abs have been reported to cooperate with suboptimal TCR stimulation in the induction of T cell proliferation [Alderson *et al.* (1993)]. Additionally, T cells from CD95 defective *lpr* mice are less responsive to antigenic stimuli than are normal T cells [Davignon *et al.* (1985)], which could be interpreted as implicating CD95 in a stimulatory role. Moreover, recent reports studying murine T cells that fail to express FADD, have shown these cells to be defective in activation induced proliferation [Zhang *et al.* (1998)]. Given that under certain circumstances CD95 may be implicated in providing a stimlatory signal to a cell, the effect of CD95 ligation on downstream targets known to be activated in T cell costimulatory signalling was investigated. However, before attempting to examine the effect of CD95 ligation on costimulatory effectors, it was important to confirm that CD95 ligation resulted in cell death by apoptosis in our T cell models. The annexin-FITC apoptosis assay kit allows detection of apoptosis at much earlier time points than previous protocols, such as those which assessed apoptosis by DNA fragmentation, since it is based on changes in the phospholipid bilayer which occur shortly after the triggering of an apoptotic stimulus [Martin *et al.* (1995)]. FITC-conjugated annexin-V was used to bind PS, a lipid normally restricted to the inner leaflet of the plasma membrane, but which is externalised as a rapid response to apoptotic signalling. Cells subjected to the annexin binding assay are analysed by FACS. Figure 6.1.1 illustrates apoptosis induction in Jurkat J6 cells in response to a four hour incubation with anti-CD95 antibody CH11. The *abscissa* represents early apoptosis as determined by annexin-FITC binding, whilst the ordinate represents plasma membrane integrity as assessed by propidium iodide exclusion. During the course of an apoptotic response, therefore, cells move from the lower left quadrant to the lower right (annexin bright), and finally undergo secondary necrosis and occupy the upper right (annexin bright, propidium iodide bright) portion of the dot-plot. Since four hours is a relatively short time point in apoptosis, the cells largely retain their membrane integrity and remain

in the lower right ("early apoptosis") region of the dot-plot. Treatment of Jurkat cells with CH11 mAb (0.1 μg/ml) induced apoptosis in 43% of the population whilst treatment of Jurkat cells with soluble Fas ligand induced a similar response with 38.9% being classed as apoptotic after four hours (Figure 6.1.1). Activation of the caspases is a general feature of apoptosis, thus the cell death observed in these experiments has been further characterised as apoptotic, by the inclusion of the pan-ICE protease inhibitor Z-VAD-FMK. Treatment of the cells with Z-VAD-FMK (30 μM) for one hour prior to incubation with CH11 was found to reduce even the basal levels of apoptosis and protect against CH11-induced apoptosis (Table 6.1). Contrastingly, treatment of cells with the PI 3-kinase inhibitors wortmannin (100 nM) and LY294002 (30 μM) was found not to inhibit, but rather potentiate apoptosis since 43.9% of Jurkat cells treated with CH11 were apoptotic after four hours, whilst populations of Jurkat cells pretreated with wortmannin and LY294002 showed 51.0% and 55.6% to be apoptotic respectively. These data suggest that apoptotic signal transduction pathways do not require activation of PI 3-kinase, but rather, PI 3-kinase may actually serve to inhibit CD95 mediated apoptosis. This observation would explain the increased susceptibility of JCaM1 cells to cell death (Table 6.2) since in JCaM 1 cells CD95 ligation was found to elicit neglible activation of PI 3-kinase as assessed by the accumulation of its lipid products (Table 6.3).



1) Control	2) CH11	3) Fas-Ligand
Cells in LR quadrant 5.02%	Cells in LR quadrant 43.12%	Cells in LR quadrant 38.9%

Figure 6.1.1 CD95 ligation by CH11 and soluble Fas-ligand induces apoptosis. Jurkat cells ($5x10^5$/point) were incubated with anti-CD95 antibody CH11 (0.1 μg/ml) (2) or soluble Fas-ligand supernatant (1:2 dilution) (3) for 4 hours. Samples were then analysed by FACS for annexin-FITC binding and propidium iodide exclusion. The control sample (1) was incubated in the absence of CH11. Data is from single representative experiment. (LR=lower right).

Treatment	% annexin positive cells
Control	10.28
ZVAD-FMK (4hr)	7.12
CH11 (4hr)	53.27
CH11 + ZVAD-FMK (4 hr)	13.77

Table 6.1 Caspase inhibitors protect against CD95 mediated apoptosis in Jurkat T cells. Control cells were incubated in medium for 4 hr. CH11 was used at 0.1 μg/ml. Where indicated, cells were pre-incubated for with ZVAD-FMK (30 μM) for one hour. Data from single representative experiment.

Treatment	% annexin v-FITC positive cells	
	Jurkat	JCaM1
Control (4hr)	12.9	25.4
CH11 (4hr)	43.9	83.5
CH11+ wortmannin	51.0	ND
CH11+ LY294002	55.6	ND

Table 6.2 Effect of PI 3-kinase inhibitors on CD95-mediated apoptosis. Control cells were incubated in medium for 4 hr. CH11 was used at 0.1 μg/ml. Where indicated cells were preincubated for with wortmannin (100 nM) or LY294002 (30 μM) for ten miutes. ND denotes not determined. Data from single representative experiment.

6.2 CD95 activates PI 3-kinase.

The enhancing effect of Fas-induced cell death by PI 3-kinase inhibition suggested that PI 3-kinase may be activated by Fas. This is particularly interesting given that PI 3-kinase activation is a critical event in T cell costimulation by CD28, and that Fas can act as a costimulatory signal under certain circumstances. Therefore, it was important to investigate the effect of CD95 ligation on PI 3-kinase activity as assessed by accumulation of PtdIns(3,4,5)P_3. CH11 (0.1 μg/ml) treatment of Jurkat cells was found to result in an accumulation of PtdIns(3,4,5)P_3 (Figure 6.2.1). However, whilst CD28 ligation was followed by a 6 to 8 fold increase over basal concentrations of PtdIns(3,4,5)P_3 (Figure 6.2.1), ligation of CD95 either by CH11 or soluble Fas Ligand was followed by a two-fold increase above basal concentrations of PtdIns(3,4,5)P_3 (Figure 6.2.1) after 10 minutes. This data demonstrates that CD95 does activate PI 3-kinase, albeit at a lower stoichiometry than CD28. Since in JCaM1 cells, CD28-mediated stimulation of PI 3-

kinase is markedly impaired in comparison to that observed in Jurkat cells, the effect of CD95 ligation on PI 3-kinase was determined in JCaM1 cells. Whilst in Jurkat cells Fas-ligand treatment was found to double cellular levels of PtdIns(3,4,5)P_3, Fas-ligand treatment of JCaM1 cells was found to have neglible effect (Table 6.3).

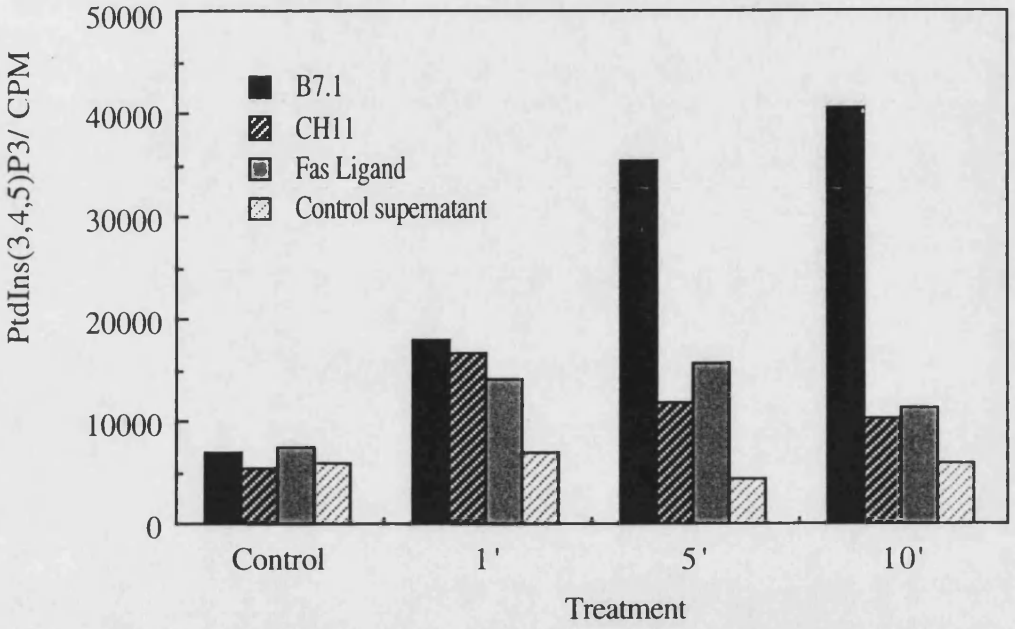


Figure 6.2.1 CD95 ligation induces PI 3-kinase activation in Jurkat cells. [^{32}P]-radiolabelled Jurkat cells ($2x10^7$) were co-sedimented with CHO-B7.1$^+$ cells (10^7), exposed to CH11 (0.1 μg/ml), soluble Fas Ligand (1:2 dilution) or control supernatant, and incubated at 37°C for the times indicated in the annotation. Cellular lipids were extracted by partitioning in chloroform, solubilised in water by deacylation and analysed by HPLC as described (see section 2.2.14). Data from single representative experiment.

Time (mins)	Fas-Ligand	
	Jurkat	JCaM1
0	6500	4321
1	7120	4967
5	19638	3867
10	12398	4086

Table 6.3 CD95 ligation stimulates negligible PtdIns(3,4,5)P_3 accumulation in JCaM1 cells. [^{32}P]-radiolabelled Jurkat or JCaM1 cells ($2x10^7$) were treated with soluble Fas Ligand (1:2 dilution) and incubated at 37°C for the times indicated. Cellular lipids were extracted by partitioning in chloroform, solubilised in water by deacylation and analysed by HPLC as described (see section 2.2.14). Data from single representative experiment.

6.3 CD95 regulates PKB activity.

From the initial observation that CD95 was capable of stimulating PI 3-kinase activity, as evidenced by increased cellular levels of PtdIns(3,4,5)P_3 (Figure 6.2.1), experiments were conducted to investigate whether CD95 activation of PI 3-kinase was sufficient to induce activation of the downstream effectors of PI 3-kinase such as protein kinase B [Burgering and Coffer (1995); Franke *et al.* (1995)]. Given the contrasting functional roles of PKB (which promotes cell survival) and CD95 (which promotes cell death), these investigations yielded some intriguing results. Figure 6.3.1 demonstrates ligation of CD95 by CH11 Ab or soluble Fas Ligand, was followed by an increase in PKB activity within a minute, which is sustained for at least five minutes following stimulation. Given that CD95 activates PI 3-kinase (Figure 6.2.1), experiments were designed to examine the potential role of PI 3-kinase in the CD95 mediated activation of PKB by incubating cells with concentrations of wortmannin, previously demonstrated to inhibit PI 3-kinase activity (see section 3.1), prior to treatment of cells with CH11. These experiments however, yielded inconclusive data as demonstrated in Figure 6.3.2. In experiment one CD95 activation of PKB appears to be inhibited by preincubation of cells with wortmannin (100 nM). Whilst in experiment two, the activation of PKB actually appears to be potentiated by the presence of wortmannin.

Given the inconsistencies noted in the generated data, further experiments were performed in an attempt to elucidate the nature of signalling pathways utilised in the CD95 mediated activation of PKB. Since MAPKAP kinase 2 is a direct substrate of p38, and has been described as potentially phosphorylating PKB on serine residue 473 [Alessi *et al.* (1996)], activation of the p38 pathway represents a potential mechanism for activation of PKB. Futher experiments were therefore performed using the inhibitor of p38 MAP kinase SB203580. Jurkat cells were treated with vehicle or with SB203580 (3-30 μM) for one hour prior to the addition of CH11 or soluble Fas Ligand. SB203580 was found to partially inhibit both CH11 and Fas Ligand-stimulated activation of PKB in a dose-dependent manner (Figure 6.3.3). Contrastingly, the activation of PKB induced by treatment of cells with 9.3 mAb was found not to be sensitive to treatment with the p38 MAP kinase inhibitor.

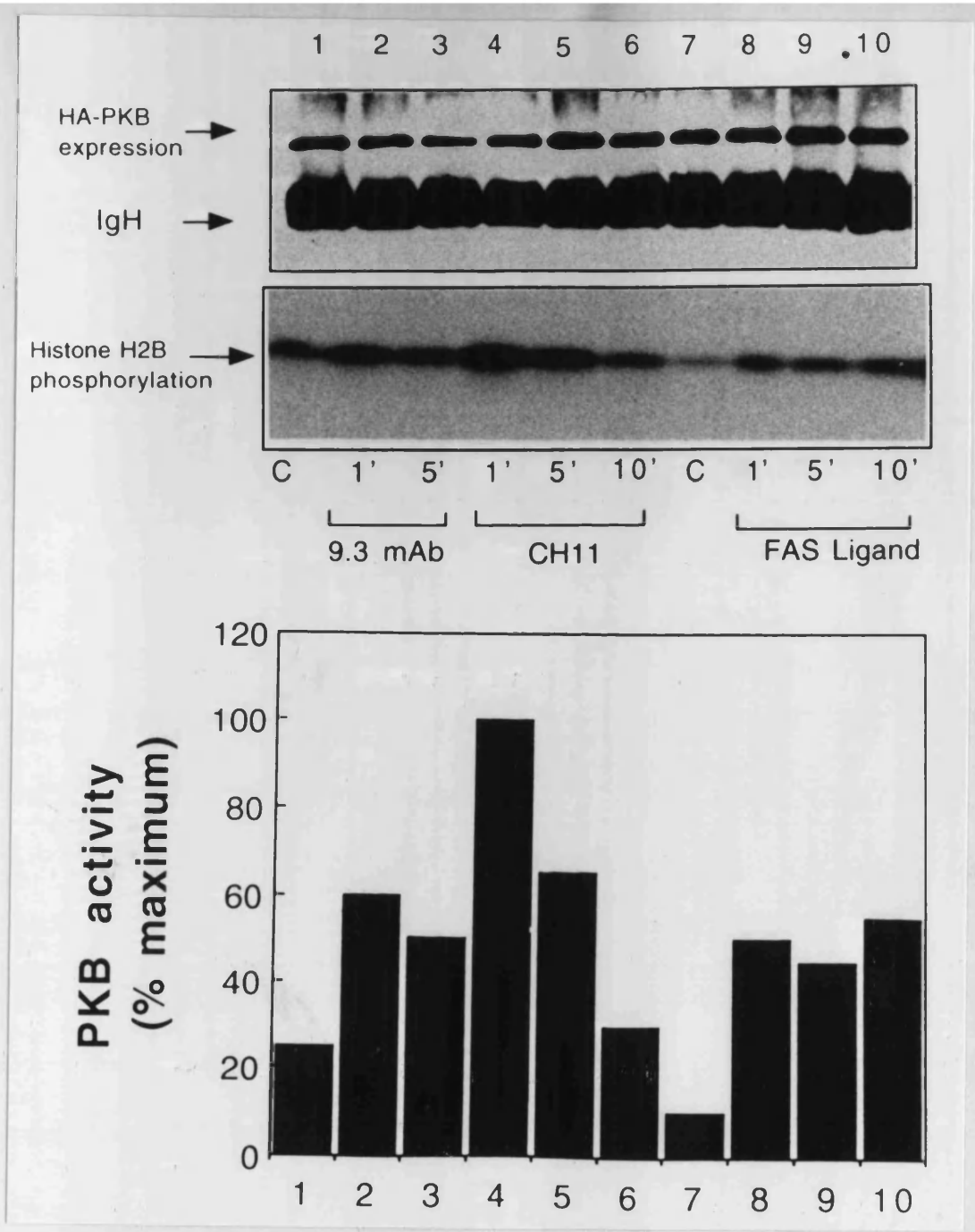


Figure 6.3.1 CD95 ligation mediates PKB activation. Jurkat cells (10^7/point) were deprived serum for 16 hours, and stimulated as detailed in the annotation. The cells were lysed and PKB immunoprecipitated using anti-PKBα antibody. Immunoprecipitates were washed as described and *in vitro* protein kinase assays performed using histone H2B as a substrate (section 2.2.18). Reactions were quenched and proteins separated by SDS-PAGE using a 7-17% acrylamide gradient gel. The lower half of the gel was dried and autoradiography performed to assess protein phosphorylation (middle panel) Densitometric analysis was then performed on the autoradiograph (lower panel). Proteins on the upper half of the gel were Western blotted to PVDF membrane and immunoblotted with anti PKBα antibody to demonstrate equal loading of protein (upper panel).

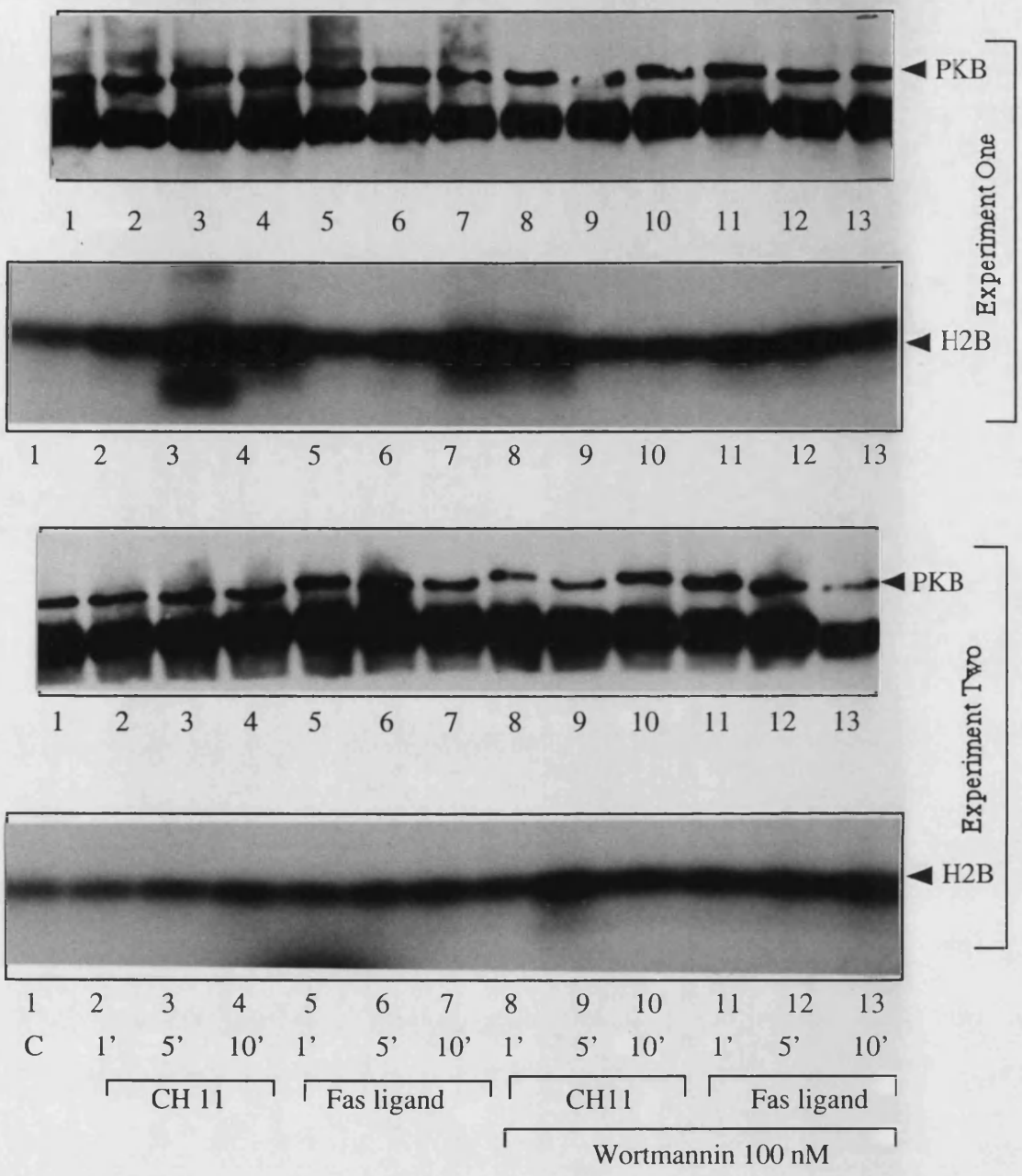


Figure 6.3.2 Effect of wortmannin on CD95-mediated activation of PKB. Jurkat cells (10^7/point) were deprived serum for 16 hours, and preincubated with wortmannin (lanes 8 to 13). Cells were then stimulated with CH11 (0.1 μg/ml) or soluble Fas Ligand (1 in 2 dilution of supernatant), as detailed in the annotation, followed by lysis in ice-cold NP40 lysis buffer. PKB was immunoprecipitated using anti-PKBα antibody. Immunoprecipitates were washed as described and *in vitro* protein kinase assays performed using histone H2B as a substrate (section 2.2.18). Reactions were quenched and proteins separated by SDS-PAGE using a 7-17% acrylamide gradient gel. The lower halves of the gels were dried and autoradiography performed to assess protein phosphorylation (lower panels). The proteins of the upper halves of the gels were transferred to PVDF membranes and immunoblot analysis carried out for the presence of PKB (upper panels). Data are from two individual experiments.

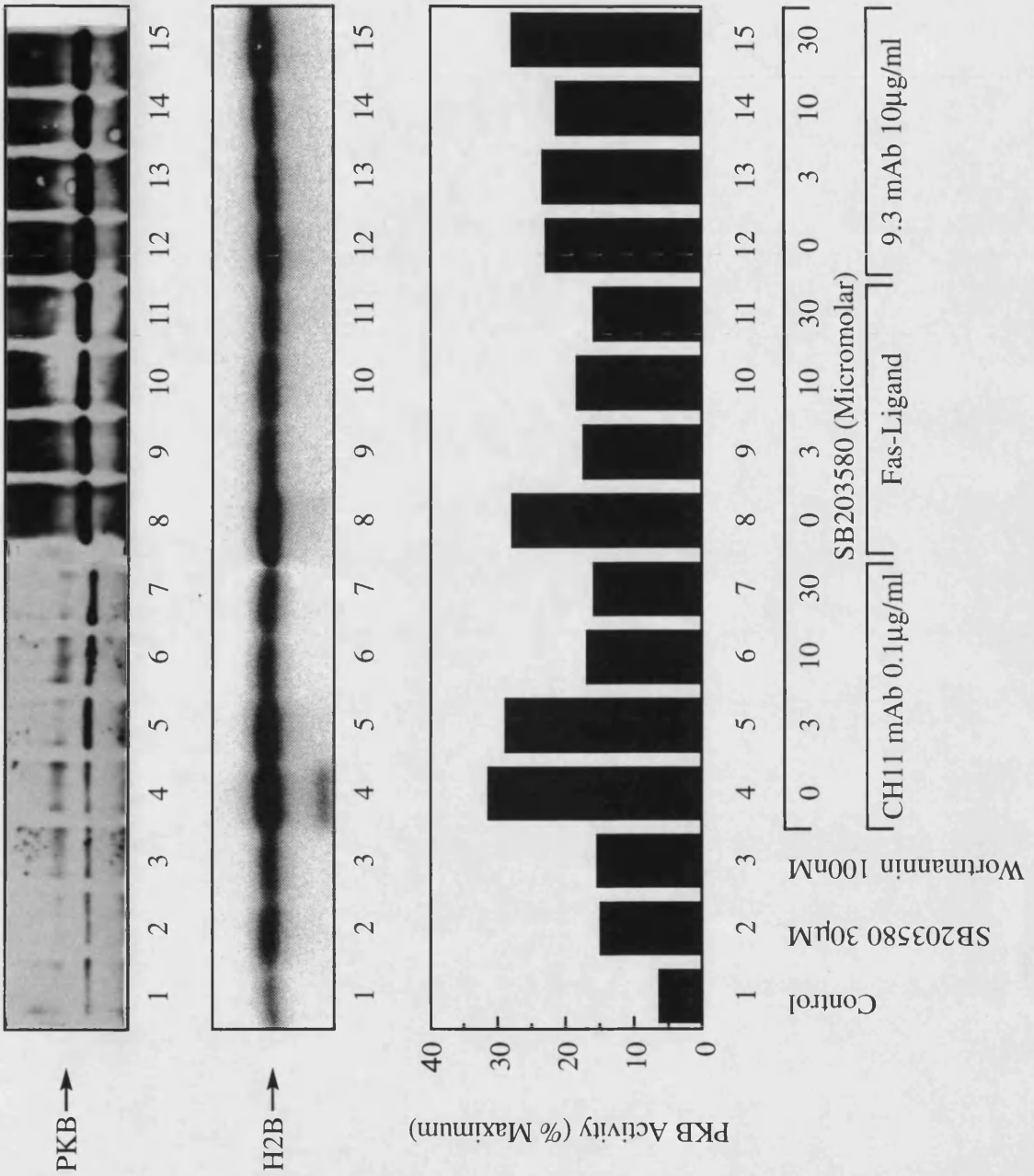


Figure 6.3.3 Effect of SB203580 on CD95-mediated activation of PKB. Jurkat cells (10^7/point) were deprived of serum for 16 hours, and preincubated with SB203580 for one hour at the concentrations described in the annotation. Cells were stimulated as described, prior to lysis in ice-cold NP40 lysis buffer. PKB immunoprecipitates, derived from the cell lysates, were washed and *in vitro* protein kinase assays performed using histone H2B as a substrate (section 2.2.18). Proteins were separated by SDS-PAGE using a 7-17% acrylamide gradient gel. The lower half of the gel was dried and autoradiographed to assess protein phosphorylation (middle panel). Densitometric analysis was then performed on the autoradiograph (lower panel). Proteins on the upper half of the gel were western blotted to PVDF membrane and immunoblotted with anti- PKBα antibody to demonstrate equal loading of protein (upper panel).

6.4 CD95 activates p38 MAP kinase.

In light of the inhibitory effect of SB203580 on CD95 mediated PKB activation, further experiments were performed to investigate the role of the p38 MAP kinase cascade in mediating the effects of CD95 on PKB. Accordingly, experiments were performed to assess whether CD95 ligation could induce p38 activation. Figure 6.4.1 illustrates p38 to be tyrosine phosphorylated at its ^{180}TGY sequence in Jurkat cells, following treatment with anti-CD95 antibody CH11 (0.1 μg/ml). This activation of p38 MAP kinase was not due to environmental stress since incubation of the cells in the absence of CH11 did not result in the phosphoryation of p38 (Figure 6.4.1, lanes 6 and 7). Maximal p38 phosphorylation however, was observed only after two hours treatment of Jurkat cells with CH11 Ab (Figure 6.4.1), thus the kinetics of p38 activation would not be suitable to support PKB activation, as described herein (Figure 6.3.1). Moreover, the activation of p38 MAP kinase, as assessed by tyrosine phosphorylation of TGY motif (Figure 6.4.1), was insensitive to the presence of the caspase inhibitor Z-VAD-FMK (30 μM), which had previously been shown to inhibit CD95-mediated apoptosis.

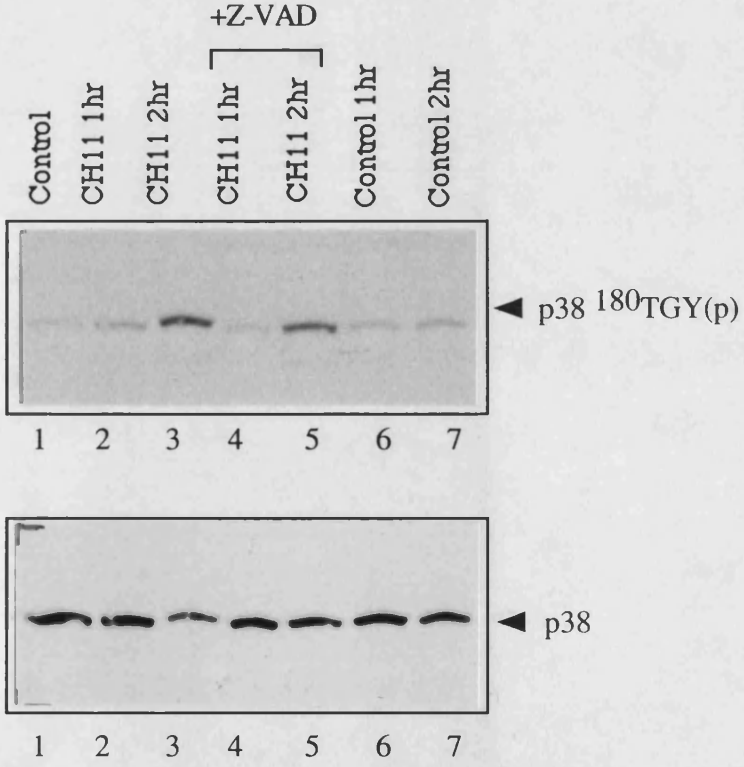


Figure 6.4.1 CD95 activates p38 MAP kinase. Jurkat cells (10^6/point) were treated as detailed in the annotation. Pretreatment with Z-VAD-FMK was for one hour at 50 μM, CH11 mAb was included at 0.1μg/ml, timed controls were incubated at 37°C for the times shown. Cells were lysed in ice-cold NP40 lysis buffer and proteins resolved by SDS-PAGE using a 10% homogeneous acrylamide gel. Proteins were transferred to nitrocellulose membranes and immunoblotted for phosphorylated p38 MAP kinase (upper panel). Blots were subsequently stripped of antibodies and reprobed for p38 MAP kinase to demonstrate equal loading of proteins.

6.5 Summary.

1) CD95 ligation by CH11 mAb and soluble Fas ligand was demonstrated to induce apoptotic cell death in Jurkat cells. Apoptosis was inhibited by preincubation of the cells with Z-VAD-FMK.

2) Treatment of Jurkat cells with CH11 mAb and soluble Fas Ligand was found to mediate activation of PI 3-kinase. Cellular levels of PtdIns(3,4,5)P_3 were found to increase up to four-fold above basal levels after 10 minutes incubation with the antibody.

3) Ligation of CD95 by CH11 mAb or Fas ligand was found to rapidly activate PKB activity in Jurkat cells for at least five minutes, as demonstrated by *in vitro* kinase assays.

4) Attempts to characterise the CD95 mediated activation of PKB yielded inconclusive results from the use of the PI 3-kinase inhibitor wortmannin. CD95 mediated activation of PKB did however, appear at least partially sensitive to inhibition of p38 MAP kinase by SB203580, suggesting a potential role for p38 MAP kinase in the CD95 mediated activation of PKB.

5) CD95 ligation was demonstrated to induce activation of p38 MAP kinase, however, maximal activation was only observed after a two hour treatment of the cells. The kinetics of CD95 mediated p38 MAP kinase activation are therefore inconsistent with those of CD95 mediated PKB activation.

SECTION SEVEN

DISCUSSION

7.1.1 CD28-mediated activation of PI 3-kinase.

Ligation of CD28 by B7.1 (this study) or anti-CD28 Abs [Pages *et al.* (1994); Prasad *et al.* (1994)] is sufficient to stimulate recruitment and activation of PI 3-kinase. In this study, ligation of CD28 has been demonstrated to stimulate *in vitro* lipid kinase activity associated with CD28 immunoprecipitates from Jurkat cells, which is presumably resultant from the activity of p110 catalytic subunits that co-associate with the CD28-bound p85 regulatory subunit, and is sensitive to inhibition by wortmannin i.e. class IA PI 3-kinases [Vanhaesebroeck *et al.* (1997)]. Analysis by *in vitro* lipid kinase assay is a useful technique since it is relatively efficient in terms of both time and use of radioisotopes, however, limitations occur in interpretation of the data generated since the assay itself imposes reaction conditions and a substrate specificity that may distort the enzyme properties. In contrast direct chloroform extraction of cellular lipids from metabolically radiolabelled cells allows for the study of changes in total phosphoinositide levels under more physiological conditions. This study has also used direct chloroform extraction of lipids therefore, to demonstrate CD28 mediated accumulation of D-3 phosphoinositide lipids in Jurkat cells.

The accumulation of the lipid products of PI 3-kinase is inhibited by preincubation of cells with wortmannin and a large part of this thesis has used wortmannin in subsequent experiments to help identify downstream PI 3-kinase-dependent biochemical events. However, problems arise in the interpretation of experiments utilising pharmacological inhibitors, since targets other than PI 3-kinase may be subject to inhibition. Wortmannin for example inhibits at least three further enzymes in PtdIns 4-kinase [Nakanishi *et al.* (1995)] and phospholipase A2 [Cross *et al.* (1995)] and mTOR [Brunn *et al.* (1995)], at concentrations coincindent with inhibition of PI 3-kinase and, at elevated concentrations, phospholipase D [Reinhold *et al.* (1990)] and phospholipase C [Bonser *et al.* (1991)]. Wortmannin inhibition of PtdIns 4-kinase could lead to disruption of the cycle of lipid phosphorylation required to replenish the PtdIns(4,5)P_2, which acts both as a substrate for phospholipase C and a precursor of PtdIns(3,4,5)P_3. Accordingly, in this study, CD28 mediated activation of PI 3-kinase has been demonstrated as inhibited by the structurally unrelated molecule LY294002, which has been previously demonstrated to inhibit purified bovine brain PI 3-kinase with an IC_{50} of 1.4 μM [Vlahos *et al.* (1994)],

whilst no significant effects of LY294002 were observed in further investigations on several enzymes including PtdIns 4-kinase, src, MAP-kinase, S6 kinase, DAG kinase, PKA and PKC [Vlahos *et al.* (1994)]. There may however be further, as yet undetermined targets for LY294002.

There are further limitations to the use of pharacological inhibitors of PI 3-kinase , for instance p85 contains two SH2 domains which may interact with tyrosine phosphorylated proteins, and an SH3 domain, which may mediate interactions with proline rich regions of proteins, additional to the p110 binding site, thus the molecule is ideally placed to perform an adaptor protein role mediating interactions with other signalling proteins. The pharmacological agents wortmannin and LY294002 inhibit only the kinase function of p110, and thus will not affect the recruitment of p85 to phosphorylated receptors or any subsequent adaptor molecule function. This must be considered when interpreting results obtained from the use of pharamacological inhibitors of PI 3-kinase such as wortmannin.

A further problem exists in interpretation of data generated using pharmacological inhibitors since these compounds inhibit all classes of PI 3-kinases to variable extents. For example wortmannin inhibits class IA PI 3-kinases and class III PI 3-kinases with an IC_{50} of <10nM [Arcaro and Wymann (1993); Volinia *et al.* (1995)], whilst the IC50 for inhibition of class II PI 3-kinase is 40-50 nM, and mammalian class II PI 3-kinases such as PI 3-kinase C2α are refractory to wortmanin [Domin *et al.* (1997)]. It is difficult therefore, to implicate class IA PI 3-kinases in a particular pathway using only such compounds. However, in the case of class IA PI 3-kinases molecular reagents have been developed which provide more definitive evidence that this class of PI 3-kinase is necessary for a particular process. Several cDNAs have been engineered to produce constitutively active or dominant negative inactive forms of PI 3-kinase, in order to demonstrate an effect of either increased PI 3-kinase activity or blocking PI 3-kinase (see section 1.5.4.3). It must be remembered however, that p85 may act as an adaptor protein with an extensive repertoire of protein-protein interactions, thus may interfere with signalling pathways by complexing with important proteins. Indeed, SHIP has been suggested to down-regulate Ras activity, following B cell antigen receptor ligation, by sequestration of the intervening adaptor protein Shc [Tridanipandi *et al.* (1997)]. Thus care must be taken in the interpretation of data derived from the use of either pharmacological or molecular reagents. Generally, the combined use of PI 3-kinase inhibitors and blocking or activation of PI 3-kinase using molecular reagents, allows for the construction of stronger arguments regarding the function of the enzyme. In this study, therefore, the CD28 mediated accumulation of the lipid products of PI 3-kinase, has been demonstrated to be resultant from the activation of class IA PI 3-kinase since it

is sensitive to inhibition by wortmannin, LY294002 and expression of Δp85. Inhibition of CD28-mediated PtdIns(3,4,5)P_3 accumulation in Jurkat cells by expression of Δp85 however, was not complete. The residual D-3 phospholipid production may be explained in a number of ways. Firstly, not all the transfected Jurkat cells might be expected to express Δp85. Hence, in cells that do not express Δp85, endogenous p85 will still be able to interact with CD28. Secondly, the residual PtdIns(3,4,5)P_3 production may be due to the activation of different classes PI 3-kinase isoforms which may have different susceptibilities to wortmannin. For example, the high affinity IgG receptor (FcγRI) couples both class IA and class IB PI 3-kinases [Melendez *et al.* (1998)].

Ligation of other cell surface antigen such as the TCR/CD3 complex, CD7 [Ward *et al.* (1995)], CD25, and signalling by growth factors, have all been reported to stimulate PI 3-kinase activity within cells. The coupling of multiple receptors to the same signalling pathway, potentially presents a problem regarding specificity of the signal delivered by these receptors. This issue may be addressed in considering a number of features of the enzyme: For instance, several biochemically distinct PI 3-kinases exist which associate with different regulatory molecules, exhibit differing substrate specificities and display varying sensitivity to inhibition by wortmannin [Table 1.1]. Moreover, diversity of class IA PI 3-kinase signalling cascade is facilitated by (i) the ability of the class I p85/p110 complex to phosphorylate three lipid substrates in PtdIns, PtdIns(4)P and PtdIns(4,5)P_2. In turn, these lipids may be compartmentalised within the cell to some degree; (ii) the p110δ isoform differs from p110α/β, in that it harbours an autophosphorylatory capacity whereas, p110α/β phosphorylate p85 [Vanhaesebroeck *et al.* (1997)], thus p110δ may potentially phosphorylate a pattern of protein substrates distinct from that stimulated by p110α/β; (iii) the SH2/SH3/proline rich regions of p85 can putatively mediate a plethora of protein-protein interactions. The diverse nature of this pathway is therefore well suited to be involved in mediating not only the diverse effects of CD28 on T cell biology, but also distinct effects of other T cell surface receptors.

7.1.2 Role of lck in CD28-mediated activation of PI 3-kinase.

The recruitment of PI 3-kinase to CD28 in the absence of lck confirms recent observations [Gibson *et al.* (1998)]. Despite the ability of lck-deficient JCaM1 cells to display ligation-dependent recruitment of PI 3-kinase to CD28, HPLC analysis of the accumulation of PtdIns(3,4,5)P_3 reveals an impaired activation of PI 3-kinase in these cells in response to CD28 ligation by B7.1. Tyrosine phosphorylation of the YMNM binding motif, within the cytoplasmic tail of CD28, is well established to be a pre-

requisite for PI 3-kinase recruitment [Pages *et al.* (1994); Prasad *et al.* (1994)]. Lck has previously been reported to be activated following CD28 ligation [August and Dupont (1994)] and to phosphorylate the PI 3-kinase binding motif in a baculovirus expression system in insect cells [Raab *et al.* (1995)]. The demonstration that PI 3-kinase recruitment to CD28 occurs in the absence of lck however, suggests that, either lck is not responsible for phosphorylation of the binding motif *in vivo* or, the absence of lck can be accommodated for by compensatory phosphorylation of the binding motif by further tyrosine kinases such as $p59^{fyn}$, ZAP 70 or ITK. The most likely of these kinases would be $p59^{fyn}$ since ITK and ZAP-70 fail to detectably phosphorylate CD28 in coexpression studies [Raab *et al.* (1995)] and optimal ITK activation is not obligatory for recruitment of PI 3-kinase to CD28 [Gibson *et al.* (1998)]. Contrastingly, GST-CD28 fusion proteins have been demonstrated to be an *in vitro* substrate for ITK immunoprecipitated from exponentially growing Jurkat cells [King *et al.* (1997)]. However, the authors also demonstrated that activation of ITK was partially dependent upon the integrity of ^{173}Tyr, implying ITK activation to be dependent upon the phosphorylation of ^{173}Tyr. Furthermore, ITK has been proposed as a lying downstream of lck and fyn in CD28 signalling [Raab *et al.* (1995)]. Thus, fyn is the strongest candidate for a CD28 ^{173}Tyr-directed kinase, in the absence of lck. Co-expression studies in insect cells however [Raab *et al.* (1995)], reveal the magnitude of CD28 phosphorylation induced by fyn to be considerably less that induced by lck, whilst the recruitment of PI 3-kinase to CD28 is unaffected by the absence of lck, as detected by Western blotting. These observations imply that there may be a further, as yet unidentified, protein tyrosine kinase which is coupled to CD28 independently of ^{173}Tyr, and required for PI 3-kinase recruitment.

The incomplete activation of PI 3-kinase in the absence of lck bears striking similarity to the previously reported mobilisation of intracellular calcium in JCaM1 cells in response to anti-CD3 antibody [Straus and Weiss (1992)]. Intracellular calcium mobilisation, a consequence of PLCγ 1 phosphorylation, occured only with slower kinetics and magnitude in cells which failed to express lck. A wild type response was restored to the JCaM1 cells on transfection of the cells with lck cDNA [Straus and Weiss (1992)], demonstrating the requirement for lck. The observation that CD28 mediated PI 3-kinase activation is incomplete in the absence of lck, implies that this kinase plays an important role in the activation of PI 3-kinase, which may not be fully compensated for by other protein tyrosine kinases. The role of lck in efficient activation of PI 3-kinase could involve either, i) direct tyrosine phosphorylation of PI 3-kinase subunits. For example, Lu *et al.* (1994) have previously correlated CD28 ligation with tyrosine phosphorylation of p110 in association with p85 which was impaired in the absence of lck. This would correlate well with the impaired activation of PI 3-kinase in JCaM1 cells described in this

study. Tyrosine phosphorylation of PI 3-kinase subunits remains a contentious issue however, since further groups have failed to detect tyrosine phosphorylation [Backer *et al.* (1992); Hu *et al.* (1992)]. Alternatively, ii) tyrosine phosphorylation of further proteins may affect PI 3-kinase function. Lck has been demonstrated to be required for CD28-stimulated phosphorylation at least two protein substrates of 97 kDa and 68 kDa [Lu *et al.* (1994)]. These proteins likely correspond to those later identified as $p97^{vav}$ and $p62^{dok}$ [Klasen *et al.* (1998)], and the efficient phosphorylation of these may be necessary for full activation of PI 3-kinase by CD28. Vav and dok may affect PI 3-kinase function as a result of enhancement of Ras activity since dok associates with Ras-GAP [Yamanashi and Baltimore (1997)], this may allow for modulation of the cellular ditribution of Ras-GAPs sequestering these negative regulators away from Ras molecules. Also, the Dbl domain of Vav has been reported to display GNEF activity for ras, at least *in vitro* [Gulbins *et al.* (1993)], however, whilst in fibroblasts PI 3-kinase binds directly to Ras resulting in activation of the lipid kinase [Rodriguez-Viciana *et al.* (1994)], a recent report suggests this pathway may not be operative in T cells [Genot *et al.* (1998)]. Lck induced phosphorylation of dok may mediate the recruitment of a further protein for example, Cbl, which associates with PI 3-kinase has been reported to associate directly with $p62^{dok}$. Recruited Cbl could enhance PI 3-kinase by allosterically modifying PI 3-kinase. The exact mechanism by which lck potentiates the CD28-mediated activation of PI 3-kinase however, remains an open question. These observations are summarised in Figure 7.1.

CD28 mediated PI 3-kinase activation in Jurkat cells appears to peak at five minutes and then diminish slightly, whilst in JCaM1 cells, lipid production appears to plateau at 5 minutes, albeit at much reduced levels compared to Jurkat. This difference may be accounted for in a number of ways. The elevated production of PtdIns(3,4,5)P_3 in Jurkat cells could lead to depletion of available lipid substrate, potentially limiting PI 3-kinase activity. Since PI 3-kinase is less active following CD28 ligation in JCaM1 cells these constraints may not be encountered. Alternatively, CD28 mediated activation of PI 3-kinase could be subject to down-regulation following lck-dependent recruitment or activation of signalling molecules that may inhibit the PtdIns(3,4,5)P_3 signal. In this respect there are two possible molecules that might be involved in negative regulation of CD28-activated PI 3-kinase. Firstly, the results presented in this study demonstrate that tyrosine phosphorylation of SHIP, which has been reported as mediated by lck [Lamkin *et al.* (1997)], is induced by CD28 ligation and correlates with an increase in SHIP catalytic activity. Thus, given that PtdIns(3,4,5)P_3 is a substrate for SHIP, lck mediated activation of SHIP may serve to limit levels of the signalling lipid, this will be discussed in more detail in section 7.3.3. Secondly, ITK is a member of the Tec family of tyrosine

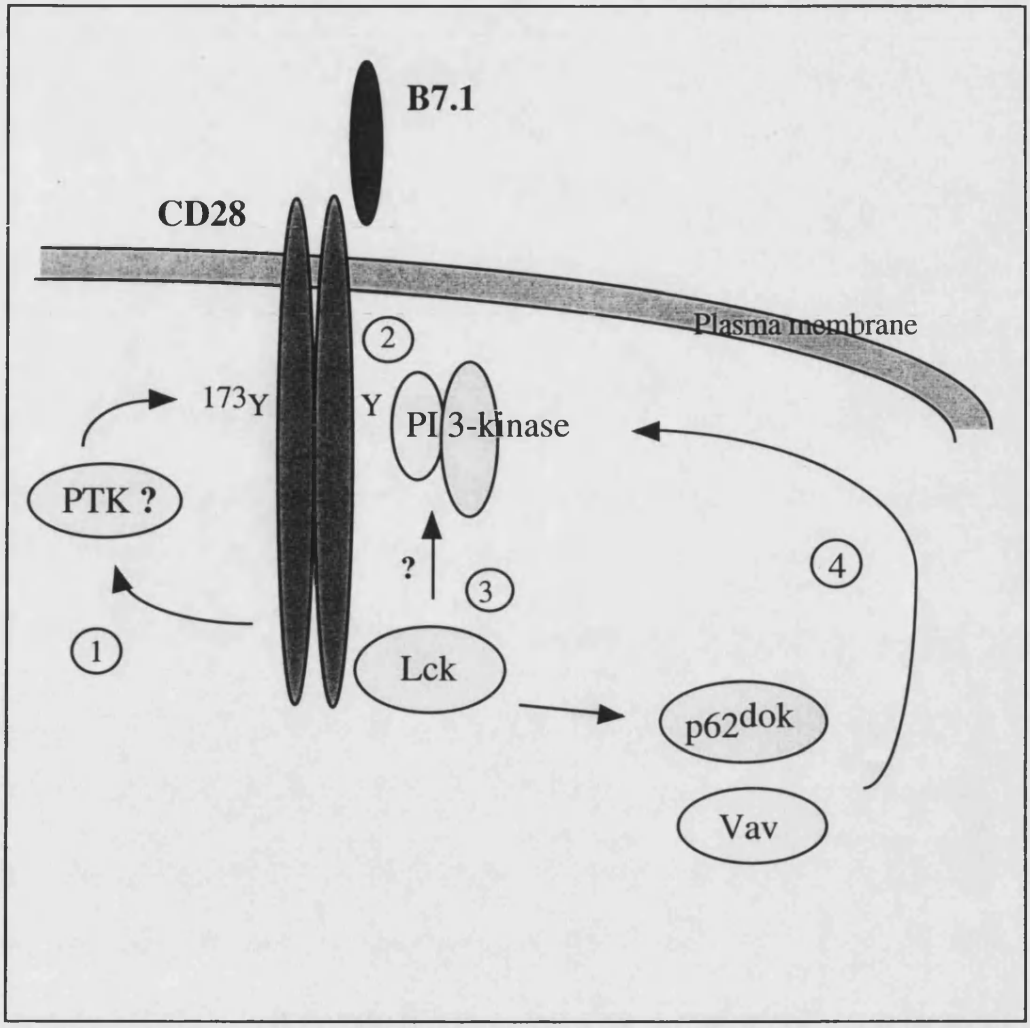


Figure 7.1 Model of CD28-mediated sequential activation of PI 3-kinase. 1) Following ligation, ^{173}Y of the CD28 cytoplasmic tail is phosphorylated. *In vitro* studies suggest lck as the strongest candidate for ^{173}Y-kinase [Raab *et al.* (1995)] however, the absence of lck does not affect PI 3-kinase recruitment to CD28, thus an unidentified kinase may mediate this phosphorylation. 2) Phosphorylation of CD28 ^{173}Y allows for PI 3-kinase recruitment via the association of CD28 with the SH2 domains of p85. 3) PI 3-kinase activtity is potentiated by lck, possibly via direct phosphorylation of the PI 3-kinase subunits or the regulation of such as Vav and p62dok [Klasen *et al.* (1998)]. 4) Vav displays GNEF activity for Ras at least *in vitro*, whilst dok may sequester the negative regulator Ras-GAP away from Ras or recruit further proteins such as Cbl which forms a complex with PI 3-kinase [Meisner *et al.* (1995)] and may allosterically modify PI 3-kinase.

kinases which associates with CD28 and becomes tyrosine phosphorylated, following ligation of the cell surface antigen [August *et al.* (1994)]. The recruitment of ITK to CD28 has been shown to be at least partially dependent on lck function [Raab *et al.* (1995); Gibson *et al.* (1996); Heyeck *et al.* (1997)]. Interestingly, proliferation studies using murine T cells defective in ITK have demonstrated that, whereas CD3 mediated proliferation was severely compromised in the absence of ITK, CD28 proliferative responses were significantly elevated, when compared with cells from control animals [Liao *et al.* (1997)]. This follows the pattern of PI 3-kinase function since the enzyme is activated by CD28, implying a costimulatory role, yet appears to play a negative role in T-cell antigen receptor function, at least in terms of NF-AT induction in Jurkat cells [Reif *et al.* (1997)]. Hypothetically, if ITK function was to inhibit the activation of PI 3-kinase, the observations of Liao *et al.* may be explained by those of Reif *et al.* Possible mechanisms for ITK modulation of PI 3-kinase may stem from the tyrosine kinase activity of the molecule. However, whilst serine phosphorylation of p85 has been reported to reduce PI 3-kinase activity by 80% [Dhand *et al.* (1994)], tyrosine phosphorylation has been reported to largely correlate with lipid kinase activity as previously discussed. Alternatively ITK mediated tyrosine phosphorylation of either p85 or any of the four tyrosine residues of the CD28 cytoplasmic tail [King *et al.* (1997)] may cause an association with a PI 3-kinase inhibitory protein via SH2 or protein tyrosine binding domains.

7.1.3 PMA disrupts CD28 coupling and activation of PI 3-kinase.

Given the presence of several potential PKC phosphorylation sites within the cytoplasmic tail of CD28, one of which is juxtapositioned to the PI 3-kinase binding site, the possibility was considered that PKC may modulate PI 3-kinase binding to the cytoplasmic tail of CD28. Immunoblotting experiments revealed that phorbol esters partially inhibited CD28: PI 3-kinase associations, and the previously reported CD28-mediated increase in PtdIns(3,4,5)P_3 [Ward *et al.* (1993)]. A number of lines of evidence suggest that PMA exerts its effect via activation of PKC: Firstly, cells are protected against the effects of PMA by pretreatment with Ro31/8220, an inhibitor of PKC. Secondly, the effects of PMA are not mimicked by the non-PKC activating phorbol ester 4α phorbol. However, PMA was found to have no reproducible effect on CD28 phosphorylation and numerous PKC inhibitors failed to prevent the ligation stimulated phosphorylation of CD28. Furthermore, transfection of constitutively active PKCα and ζ isoforms was without effect upon the basal CD28-induced lipid kinase activity of PI 3-kinase in Jurkat cells, although we cannot be sure that these proteins were expressed. Hence, the observed inhibitory effect of PMA on CD28: PI 3-kinase activation may be

explained by effects of PMA-activated PKC on PI 3-kinase itself, rather than CD28. Certainly the δ and ε isoforms of PKC contain consensus sequence-recognition motifs for p85 SH2 domains and associate with PI 3-kinase [Ettinger *et al.* (1996)]. Moreover, PMA treatment stimulates phosphorylation of the p110 catalytic and p85 regulatory subunits on serine and threonine residues respectively [Reif *et al.* (1993)]. Since the p85 subunit may act as a multi-functional adaptor molecule by virtue of its dual SH2, SH3 and/or proline rich domains, PMA stimulated phosphorylation of the p85 subunit may disrupt protein-protein interactions involving the p85 subunit. Alternatively, the reported PMA-stimulated serine phosphorylation of the p110 subunit may modulate PI 3-kinase activity. Certainly, PMA can stimulate modest accumulation of PtdIns(3,4,5)P_3 in fibroblasts [Petrisch *et al.* (1995)] and platelets [Zhang *et al.* (1996)]. However, neither PMA nor PKC inhibitors have any significant effect on the basal levels of PtdIns(3,4,5)P_3 in Jurkat cells. This is an unexpected observation given that $p21^{ras}$ is a potent downstream target of phorbol ester-activated PKC in T cells [Izquierdo *et al.* (1992)], and in other systems $p21^{ras}$ can interact directly with the p110 catalytic subunit [Rodriguez-Viciana *et al.* (1996)] and has been reported to activate PI 3-kinase [Rodriguez-Viciana *et al.* (1994)]. Thus T cells may express distinct isoforms of PI 3-kinase whose activity is independent of a regulatory input from $p21^{ras}$. Our data is consistent with the recent observations of Genot *et al.* (1998), who demonstrated that $p21^{ras}$ initiates Rac-1 but not PI 3-kinase/PKB mediated signalling pathways in T lymphocytes. Hence, the PI 3-kinase mediated signalling cascade is not a universal Ras effector molecule. The role of PKC in mediating inhibition of CD28-stimulated PI 3-kinase activation is represented in a speculative model in Figure 7.2.

Another unexpected observation was that although treatment with the PKC inhibitor Ro31/8220 almost fully prevented PMA stimulated down-regulation of p85 association with CD28, the levels of lipid kinase activity associated with CD28 immunoprecipitates were not similarly restored. The reason for this discrepancy is not clear, however this observation may reflect the differential involvement of various PKC isozymes in the regulation of PI 3-kinase/CD28 protein interactions and/or PI 3-kinase activity. Although these isozymes would be stimulated by PMA under the conditions employed in this study, they may exhibit different sensitivity to the PKC-inhibitor Ro31/8220 and this may be a possible explanation of these observations. Alternatively, this discrepancy may represent an *in vitro* artifact, since the PMA-stimulated down regulation of *in vivo* CD28-stimulated PtdIns(3,4,5)P_3 accumulation that was observed in [^{32}P]Pi-labelled cells was almost totally prevented by pretreatment with Ro31/8220.

Phorbol ester disruption of CD28 mediated PI 3-kinase activation has been reported by

other groups [Hutchcroft *et al.* (1995)] who ligated CD28 with mAbs. The work presented in this study has used the natural ligand B7.1 to stimulate CD28, an important consideration given the reported discrepancies between signals generated by mAb ligation and B7.1 in CD28 mediated activation of $p21^{ras}$ and PLC signalling pathways [Nunes *et al.* (1994)]. The inhibitory effects of PKC activation on CD28 coupling to PI 3-kinase may operate to limit CD28 signal transduction, and the physiological signal may be elicited by CD28 as part of a negative feedback mechanism. Indeed levels of diacylglycerol have been demonstrated to be elevated following CD28 ligation [Nunes *et al.* (1993)]. Alternatively, molecules such as the TCR/CD3 complex which activate PKC [Nishizuka (1988)] may mediate a possible cross talk mechanism that modulates CD28 signalling.

Previous studies have reported that wortmannin does not inhibit IL-2 production from Jurkat after combined treatment with PMA and anti-CD28 mAb [Hutchcroft *et al.* (1995)], implying that PI 3-kinase is not necessary for lymphokine production. However, leukaemic T cells may not be good functional models of T cell costimulation since these cells have been selected in culture to grow and proliferate in the absence of any costimulatory signal. Indeed, when peripheral blood-derived T cells are used as a model, nanomolar concentrations of wortmannin do inhibit both [^{3}H]-thymidine incorporation and IL-2 production induced by the combination of cross-linked anti-CD3 mAb and B7.1 [Ward *et al.* (1995)]. Furthermore, the structurally unrelated and more specific PI 3-kinase inhibitor LY294002, has similar effects on CD28 driven IL-2 production from normal T cells [Ueda *et al.* (1995)] indicating that the effects of wortmannin are unlikely to be due to the reported non-specific actions on other kinases. This study demonstrates that both PI 3-kinase inhibitors can also inhibit, albeit partially, normal T cell incorporation of [^{3}H]-thymidine induced by CD28 ligation in the presence of 0.5 ng/ml PMA, a concentration that is not sufficient to disrupt PI 3-kinase coupling to CD28. In contrast, a previous study demonstrated that IL-2 production from normal T cells driven by sub-nanomolar concentrations of PMA and the CD28 mAb 248 was resistant to wortmannin [Ghioti-Ragueneau *et al.* (1996)]. This study has demonstrated that PI 3-kinase inhibitors can prevent the [^{3}H]-thymidine incorporation (and presumably IL-2 production) induced by B7.1, in the presence of low concentrations of PMA. The reason for this discrepancy is not clear although previous studies have indicated that qualitatively different signals can be transmitted through CD28 by a panel of different CD28 mAbs and certainly, the CD28 mAb 248 is able to support IL-2 production in the absence of PMA [Nunes *et al.* (1993)].

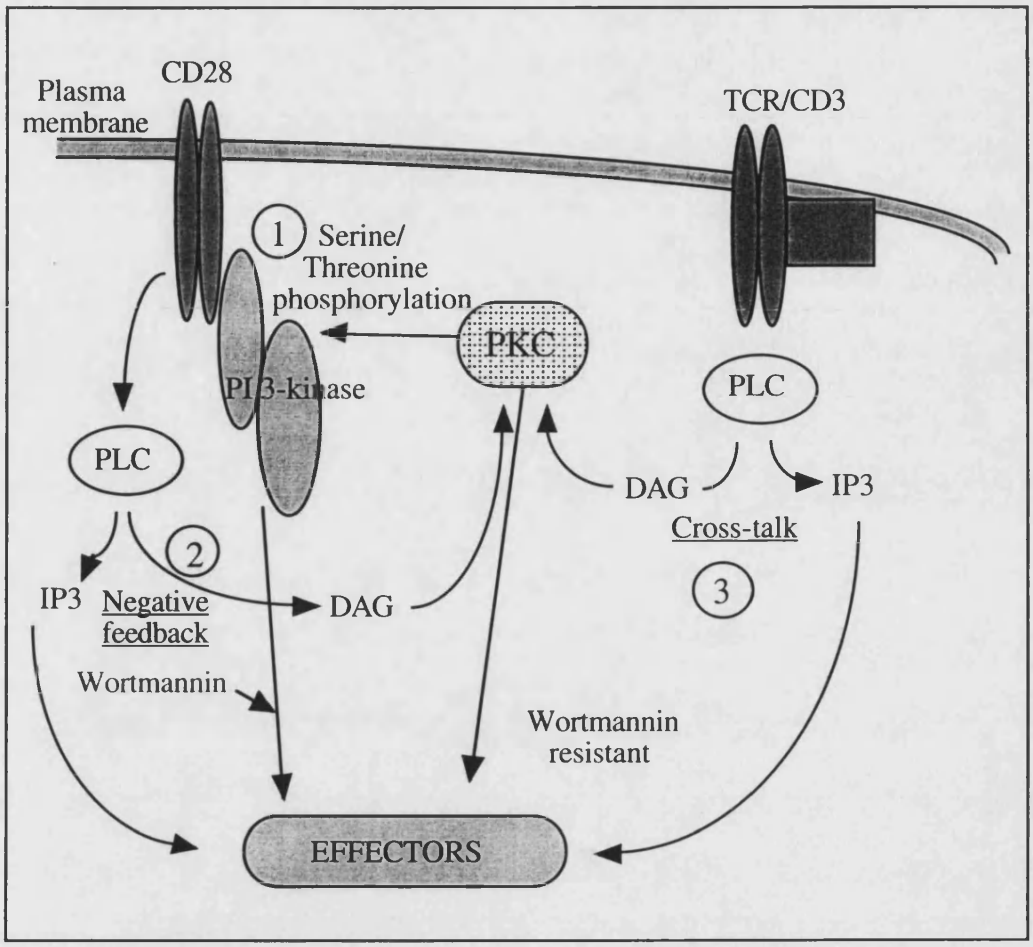


Figure 7.2 Model for PKC-mediated down regulation of CD28-activated PI 3-kinase signalling. 1) Since both PMA and PKC inhibitors were without effect on the phosphorylation profile of CD28, the observed inhibitory effect of PKC on PtdIns(3,4,5)P_3 accumulation may be a consequence of a direct effect on PI 3-kinase. Indeed, PMA has been previously reported to induce threonine and serine phosphorylation of p85β and p110, respectively [Reif *et al.* (1993)]. 2) PKC may be activated by CD28 derived signals as a negative feedback mechanism or 3) TCR/CD3 signalling as a cross-talk mechanism.

Interestingly, [^{3}H]-thymidine incorporation driven by CD28 ligation in the presence of higher concentrations of PMA (5-50 ng/ml) is markedly reduced in comparison to responses observed in the presence of lower concentrations of PMA. These functional observations in the presence of 5-50 ng/ml PMA correspond with the inhibitory actions of these concentrations of PMA on CD28 coupling to PI 3-kinase. Surprisingly the remaining responses to CD28 and higher concentrations of PMA appeared to be either less sensitive or completely resistant to PI 3-kinase inhibitors, despite the demonstration that a substantial component of the (approximately 20-30%) of the PI 3-kinase coupled to CD28 is unaffected by the phorbol ester treatment. A possible explanation for this

observation is that higher concentrations (>5 ng/ml) of PMA are sufficient to provide post-receptor activation of the downstream targets of PI 3-kinase. Thus, T cell functions determined in the presence of higher concentrations of PMA would be expected to be resistant to PI 3-kinase inhibitors. Indeed several of the known and putative targets of PI 3-kinase are activated by PMA such as the phorbol ester-sensitive PKC isoforms δ,ε,η [Toker *et al.* (1994)] as well as p70 S6 kinase [Chung *et al.* (1994)] and the ERK and JNK pathways [Nunes *et al.* (1996)], thus, the concentration of PMA appears to be critical in determining whether or not the resulting CD28-dependent T cell responses are sensitive to PI 3-kinase inhibitors.

7.2.1 Ligation-dependent phosphorylation of CD28 is insensitive to pharmacological inhibitors of serine/threonine kinases.

The data presented in this study shows that stimulation of CD28 by its natural ligand B7.1 results in heavy phosphorylation of CD28 on serine and threonine residues. The presence of several potential PKC phosphorylation sites [Nishikawa *et al.* (1997)] suggested that the observed serine/threonine phosphorylation of CD28 in response to B7.1 ligation might be mediated by PKC phosphorylation of the CD28 cytoplasmic tail. Indeed, PMA has been reported to induce the phosphorylation of CD28 exclusively on threonine residues using an *in vitro* immune complex assay, which detected activity of a threonine kinase co-associated with CD28 immunoprecipitates [Hutchcroft *et al.* (1996)]. Since the conditions employed were not permissive to PKC activity, it was proposed that PKC induces *in vivo* phosphorylation of CD28 by one or more PKC isoforms, which in turn allows recruitment and activation of a kinase responsible for the *in vitro* phosphorylation of CD28 [Hutchcoft *et al.* (1996)]. It is possible however that the threonine kinase detected by immune complex assays may not be functional under *in vivo* settings and may be physiologically redundant. Indeed, in this study using a metabolic labelling approach with ^{32}P, no direct effect was detected of PMA on CD28 phosphorylation in Jurkat cells. Moreover, several known inhibitors of PKC had no effect upon ligation-dependent phosphorylation of CD28. The discrepancies between findings in this study and the observations of Hutchcroft *et al.* may be explained in a number of ways. The study that demonstrated PMA-induced threonine phosphorylation utilised the mild detergent Brij 96 in cell lysis buffers [Hutchcroft *et al.* (1996)]. Brij 96 has previously been used to maintain CD3-PTK associations which are otherwise lost using more stringent detergents such as NP40 [Beyers *et al.* (1992)]. Secondly, the use of an *in vitro* immune complex assay system may preferentially support threonine kinase activity that is not active under normal *in vivo* conditions, leading to the detection

of *in vitro* artifacts. Thirdly, the *in vivo* metabolic labelling approach adopted in this study may allow CD28 to be subjected to specific protein phosphatase(s) which target the PKC phosphorylation motif. These phosphatases may be lost during cell lysis and therefore would not be operative under *in vitro* immune complex assay conditions. Finally, it is possible that the differences between observations in this study and that of Hutchcroft *et al.* may be explained by the use of different Jurkat subclones, which may display biochemical differences.

In an attempt to characterise the nature of the CD28 targeted serine/threonine kinase, radiolabelled cells were preincubated with pharmacological agents reported to influence the activity of known serine/threonine kinases. Since both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) are intracellular second messengers which display serine/threonine kinase activity, cells were incubated with cell permeable stable analogues of cAMP or cGMP namely, Rp-cAMPs, 8-bromo-cAMP or 8-bromo-cGMP, to assess their effect on basal and B7.1-stimulated CD28 phosphorylation. Although CD28 has not been demonstrated to activate PKA, CD28 costimulation has been reported to activate cyclic AMP-responsive element binding proteins in T lymphocytes [Hsueh *et al.* (1997)], implying some role for PKA in costimulatory signalling. Additionally, PKA has been implicated in the phosphorylation of another T cell surface antigen, namely CD27 [Sugita *et al.* (1997)] which, analagous to CD28, lacks any consensus motif for phosphorylation by PKA (RXXS). Thus neither CD27 nor CD28 could constitute a direct substrate for phosphorylation by PKA. In contrast to cAMP, CD28 has been reported to elevate levels of cGMP [Ledbetter *et al.* (1986)], suggesting that PKG may be activated subsequent to CD28 ligation. No effects however, were detected either on the basal phosphorylation state of CD28, or following B7.1 stimulation following treatment of cells with pharmacological agents known to affect the activity of PKA or PKG. These data therefore argue against the involvement of either PKA or PKG in the phosphorylation of CD28.

The ^{177}Thr residue of the CD28 cytoplasmic domain, lies within a potential site for phosphorylation by the proline directed serine/threonine kinases such as ERK [Davis (1994)]. ERK has been shown to phosphorylate Sos and down-regulate the Ras activation pathway in a proposed negative feedback mechanism [Buday and Downward (1995)]. Additionally a striking analogy can be drawn between CD28-mediated signal transduction and feedback mechanisms proposed to operate in insulin signalling [Shepherd *et al.* (1998)]. Mutational analysis suggests that some or all of the serine residues (^{612}Ser, ^{632}Ser, ^{662}Ser and ^{731}Ser) within IRS-1 participate in the negative regulation of insulin stimulated PI 3-kinase activity induced by the phosphatase inhibitor

okadaic acid [Mothe and Van Obberghen (1996)]. These residues all lie in consensus motifs for MAP kinase phosphorylation (PXSP), in close proximity to the YMXM tyrosine phosphorylation site of IRS-1. Since MAP kinase is activated by insulin in parallel with PI 3-kinase, this has been proposed as a basis for cross talk between the two pathways, leading to feedback regulation of PI 3-kinase activity [Shepherd *et al.* (1998)]. However, there are several reasons why it is unlikely that the known members of the MAP kinase family such as ERK, JNK and p38 MAP kinase are involved in mediating ligation-stimulated CD28 phosphorylation. Firstly, treatment of cells with the MEK inhibitor PD98059 had no effect upon the phosphorylation of CD28 induced by B7.1. Secondly, ligation of CD28 by B7.1 does not stimulate the ERK cascade [Nunes *et al.* (1994)] and hence would not be activated under the conditions employed in the assay. Similarly, it is unlikely that JNK subgroups of the MAP kinase family would mediate ligation stimulated CD28 phosphorylation since CD28 signals alone are insufficient to activate JNK, but instead require the additional presence of signals provided by either PMA/ionomycin or CD3 ligation [Su *et al.* (1994); Nunes *et al.* (1996)]. Finally, although we have shown that CD28 can induce modest activation of p38 MAP kinase, it is unlikely that p38 is involved in the phosphorylation of CD28 since the kinetics are much slower than B7.1-stimulated CD28 phosphorylation, and the p38 MAP kinase inhibitor SB203580 does not inhibit ligation-dependent CD28 phosphorylation.

Ligation of CD28 is known to be followed by ceramide generation via the activation of acidic sphingomyelinase [Boucher *et al.* (1995)], downstream effector targets of which are known to include serine/threonine kinase activity in the form of ceramide activated protein kinase (CAPK). It was therefore important to investigate whether CD28 was a target for ceramide-activated kinases by treatment of cells with ceramide analogues C_2 ceramide, and the biologically inactive C_2 dihydroceramide [Westwick *et al.* (1995)]. Both compounds were found to be without effect either on the basal, or the B7.1 stimulated, phosphorylation of CD28. These data therefore strongly argue against the involvement of ceramide-activated kinases in the phosphorylation of CD28.

It is known that CD28 activates PTKs such as lck, fyn and ITK, and can act as a target for these PTKs. We postulated therefore, that the tyrosine phosphorylation may be required for the recruitment of CD28-directed serine/threonine kinase, either by direct interactions with phosphotyrosine residues, or indirectly via adaptor molecules. To determine if there was a requirement for activation of *src* family tyrosine kinases in the serine/threonine phosphorylation of CD28 therefore, Jurkat cells were incubated overnight with the inhibitor Herbimycin A, before co-sedimentation with CHO-B7.1$^+$

cells. Herbimycin A treatment of the cells resulted in a marked inhibition of B7.1 stimulated CD28 phosphorylation. This experiment is open to a number of criticisms: i) the experiment was not repeated due to a lack of available inhibitor, ii) CD28 expression was not determined after incubation with Herbimycin, and iii) the observed effects may be due to an inhibition of cellular metabolism and hence ability to assimilate the radiolabel. However, taken with the results from CD28 mutation studies to be discussed later (section 7.2.3), this experiment provides evidence for *src* kinase function as a pre-requisite to serine/threonine phosphorylation of CD28. A possible explanation for this observed dependance upon Tyr phosphorylation of CD28 could lie in the ability of ^{173}Tyr to recruit adaptor molecules. Both p85 and Grb-2 associate with the phosphorylated YMNM motif within the CD28 cytoplasmic tail, and this may allow for recruitment of serine/threonine kinases. For example, Grb-2 has been reported to associate with the T cell antigen receptor on T cell activation and recruit a serine/threonine kinase via SLP-76, in Jurkat cells [Motto *et al.* (1996)]. Thus recruitment of a serine/threonine kinase appears to be dependent upon the prior activity of a tyrosine kinase. However, it should be noted that Herbimycin A is a fairly non-selective agent since it induces non-specific covalent modification of thiol groups, thus this data could reflect non-selective inhibition of the serine/threonine kinase activated by CD28 ligation [Mahon and O'Neill (1996)].

Since the serine/threonine phosphorylation of CD28 is a ligation-dependent event, it may be assumed to carry some physiological relevance. However, in the absence of any effective inhibitory strategy, it is difficult to determine whether serine/threonine phosphorylation of CD28 would positively or negatively regulate CD28-mediated activation of PI 3-kinase. Thus it is valid to postulate that serine/threonine phosphorylation may serve to enhance PI 3-kinase activity by stabilising protein-protein interactions. Striking analogy may be drawn however, with the IRS-1 mediated activation of PI 3-kinase. The IRS-1 protein recruits PI 3-kinase via a YMXM motif, whilst phosphorylation of some, or all, of the ^{612}Ser, ^{632}Ser, ^{662}Ser and ^{731}Ser residues of IRS-1 negatively modulates insulin-stimulated PI 3-kinase activity.[Mothe and Van Obberghen (1996)]. Assuming similar mechanisms to be applicable to CD28-activated PI 3-kinase, serine/threonine phosphorylation of CD28 may serve as a receptor-operated negative feedback inhibition mechanism. This hypothesis is summarised in Figure 7.3.

7.2.2 Which regions of the CD28 cytoplasmic tail are required for ligation-dependent phosphorylation.

The experiments performed with the CD28 deletion mutants demonstrate that the integrity of the PKC phosphorylation motif around ^{177}Thr and/or the ^{173}YXXM motif is required for optimal ligation-induced CD28 serine/threonine phosphorylation. It should be remembered that the non-specific nature of deletion mutation may mean several sites that may be of importance to CD28 phosphorylation may be disrupted. For example, the Δ21 mutant partially disrupts one proline rich motif (^{178}Pro-Arg-Arg-Pro) and completely deletes another (^{190}Pro-Tyr-Ala-Pro) (see Figure 2.1). Thus the reduced phosphorylation of CD28 in the Δ21 mutant may be as a result of impaired recruitment of serine/threonine kinase(s) via the proline rich regions. Three Tyr residues (^{188}Tyr, ^{191}Tyr and ^{200}Tyr), as well as the ^{184}Thr (which lies within a potential PKC phosphoylation site) and ^{202}Ser are also deleted in the Δ21 mutants and this may account for the small reduction in the overall phosphorylation profile of CD28 in Δ21 compared to wild types controls. Certainly, site-specific mutagenesis studies have revealed that integrity of the ^{188}Tyr and ^{200}Tyr residues is necessary for costimulation of IL-2 production indicating their importance to CD28 signalling [Pages *et al.* (1996); Truitt *et al.* (1996)]. Although ligation-induced tyrosine phosphorylation of CD28 can be detected by immunoblotting with anti-phosphotyrosine mAb [Pages *et al.* (1994)], phosphoamino acid analysis of CD28 immunoprecipitated from [^{32}P]-labelled Jurkat cells has revealed tyrosine to form only a small percentage of the overall phosphorylation that can be detected. Thus, tyrosine phosphorylation may occur at very low stoichiometry relative to serine/threonine phosphorylation and it therefore seems unlikely that deletion of ^{188}Tyr, ^{191}Tyr and ^{200}Tyr in Δ21 contributes significantly to reduction in total CD28 phosphorylation detected in CD28 immunoprecipitates derived from T cell hybridomas. Surprisingly, ligation-induced phosphorylation was not detected in the Δ30 mutant, in which the motif around ^{177}Thr, as well as the (p)^{173}Tyr-Met-Asn-Met motif responsible for binding PI 3-kinase, are additionally deleted. The Δ30 mutant contains three serine residues (^{163}Ser which lies within a potential PKC phosphorylation site, as well as ^{166}Ser and ^{171}Ser) which might be expected to act as targets for serine kinases since phosphoamino acid analysis revealed approximately equal quantities of phosphoserine and phosphothreonine. One reason for the failure to detect phosphorylation of CD28 in Δ30 mutants, may be that the regions distal to ^{172}Asp (e.g. the proline rich regions and the tyrosine residues at positions 173, 188, 191 and 200) may be required for recruitment and/or activation of the serine/threonine kinase(s) responsible for the putative phosphorylation of these residues as already discussed.

7.2.3 The involvement of PI 3-kinase in CD28 phosphorylation.

Interestingly, PI 3-kinase has previously been demonstrated to act as both a lipid kinase and a protein serine/threonine kinase [Dhand *et al.* (1994)]. Indeed site-specific mutagenesis of the ^{173}Tyr, which disrupts PI 3-kinase binding, results in complete loss of detectable serine/threonine phosphorylation and has been previously demonstrated to correlate with an 80% reduction of detectable CD28 tyrosine phosphorylation [Pages *et al.* (1994)] suggesting that the integrity of this PI 3-kinase binding motif is required for optimal phosphorylation. Therefore, PI 3-kinase may mediate B7.1 stimulated CD28 phosphorylation, since it is rapidly and strongly recruited to CD28 following ligation. However, site-specific mutation of ^{200}Tyr, which is sufficient to reduce the association of CD28 with PI 3-kinase by 80%, has no effect upon ligation induced tyrosine phosphorylation of CD28 [Pages *et al.* (1996)] and ligation-stimulated phosphorylation of CD28 is comparable in the DYF200 mutant to that observed in wild type CD28 expressing cells. Nevertheless, whilst binding of PI 3-kinase to CD28 is reduced in the DYF200 CD28 mutant [Pages *et al.* (1996)] the amounts of PI 3-kinase bound to CD28 may still be sufficient to mediate normal serine/threonine phosphorylation of CD28 following ligation by B7.

Compelling evidence against a role for PI 3-kinase in CD28 phosphorylation was obtained from the experiments utilising wortmannin and LY294002, which failed to abrogate the ligation-stimulated phosphorylation of CD28. There are however, certain problems with the interpretation of the results obtained with PI 3-kinase inhibitors. For instance, it has previously been demonstrated that wortmannin does not prevent the ligation stimulated recruitment of PI 3-kinase to CD28 [Ward *et al.* (1995)]. Thus one possibility is that the inhibitors were not sufficiently effective to prevent CD28-associated PI 3-kinase activity. This remaining PI 3-kinase activity may be below the sensitivity of the assays employed to detect lipid kinase activity, but sufficient for maximal stimulation of CD28 phosphorylation. Furthermore, the proposed adaptor molecule function of the p85 subunit of PI 3-kinase may mediate the recruitment of a serine/threonine kinase responsible for phosphorylating CD28. This function of PI 3-kinase would be insensitive to PI 3-kinase inhibitors, but would be disrupted by mutagenesis of ^{173}Tyr. Alternatively the ubiquitous adaptor molecule Grb-2, which contains SH2 and SH3 domains, has also been reported to bind to CD28 via the motif around ^{173}Tyr, albeit at much lower stoichiometry than observed for PI 3-kinase [Schneider *et al.* (1995a)], and may be involved in the recruitment of a serine/threonine

kinase responsible for phosphorylation of CD28. In contrast with the effects on PI 3-kinase, it is possible that mutation of ^{200}Tyr has no effect on Grb-2 association with CD28, which would explain the lack of effect of this mutation on CD28 phosphorylation. The involvement of CD28 ^{173}Y binding adaptor proteins in CD28 serine/threonine phosphorylation is represented in Figure 7.3.

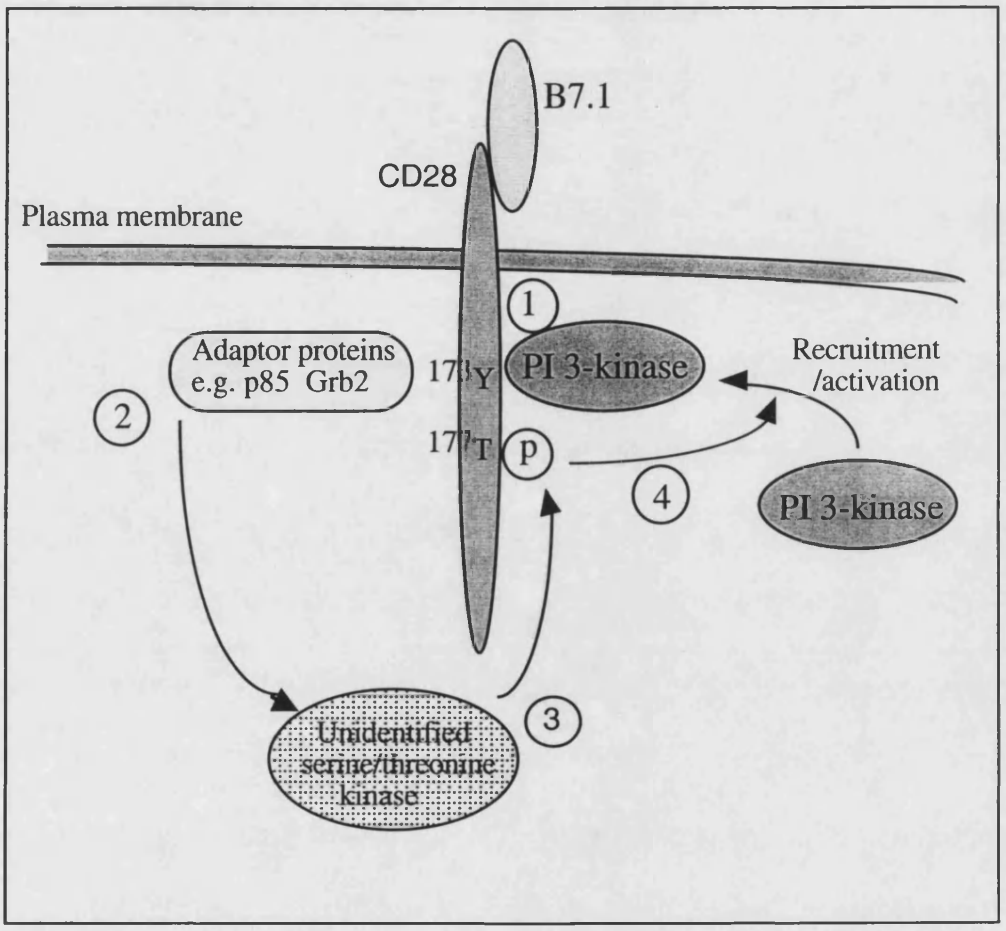


Figure 7.3 Proposed receptor-operated negative feedback regulation of PI 3-kinase. Since serine/threonine phosphorylation of CD28 is a ligation-dependent event it may be assumed to carry physiological relevance. By analogy with IRS-1 activation of PI 3-kinase, where serine phosphorylation of IRS-1 negatively regulates PI 3-kinase activity, this may represent a negative feedback mechanism, as follows: 1) Ligation of CD28 results in recruitment and activation of PI 3-kinase. 2) Adaptor molecules recruited to CD28 via SH2, SH3 and/or proline rich interactions recruit unidentified serine/threonine kinase(s). 3) CD28 is phosphorylated on serine/threonine residues. ^{177}Thr may represent an important site since (i) it is proximal to the PI 3-kinase binding site of CD28, and (ii) deletion mutation of this residue abolishes detectable serine/threonine phosphorylation of CD28. PI 3-kinase does not mediate serine phosphorylation of CD28 since pharmacological inhibitors of PI 3-kinase do not affect CD28 phosphorylation. The identity of the serine/threonine kinase responsible for phosphorylation of CD28 is not clear since this phosphorylation is resistant to a panel of inhibitors of known serine/threonine kinases. 4) serine/threonine phosphorylation of CD28 inhibits activation and/or futher recruitment of PI 3-kinase.

7.3.1 CD28-mediated activation of downstream effectors and regulators of PI 3-kinase-dependent signalling cascades: Protein kinase B.

The work detailed in section 5.3 constitutes the first demonstration that ligation of CD28 by either anti-CD28 mAb or the natural ligand B7.1 results in the activation of the PI 3-kinase downstream effector PKB in both the leukaemic cell line Jurkat and freshly isolated normal T lymphocytes. Additionally, this activation of PKB is seen to be inhibited by preincubation of the cells with wortmannin. The effects of ligation by B7.1 are demonstrated as specific, since B7.1 induced PKB activation could also be prevented by prior incubation of the CHO-B7.1$^+$ cells with CTLA4-Ig fusion protein. Since the CD28-mediated activation of PI 3-kinase is well established [Ward *et al.* (1993)], these data strongly suggest that PKB is likely to be a downstream component of the CD28 activated PI 3-kinase dependent signalling cascade. This correlates well with the identification of PKB as a downstream effector of PI 3-kinase [Burgering and Coffer (1995)] and the reported CD28-mediated activation of PI 3-kinase in response to Ab and B7 ligation [Ward *et al.* (1993); Pages *et al.* (1994)].

Immunoprecipitation of transiently expressed HA-tagged PKB from metabolically radio-labelled Jurkat cells demonstrates that PKB is phosphorylated by a constitutively active PI 3-kinase. An unidentified phosphorylated protein of approximately 180 kDa was observed to co-imunopreciptate with HA-PKB, and this may represent a PKB-associated protein tyrosine kinase or unidentified PKB substrate.

The demonstration that CD28 ligation results in the activation of PKB has several implications. The fact that CD28 can couple to PKB is highly relevant to the protective effects of PKB against cell death, given the recent insights into the function of PKB in promoting cell surival [Dudek *et al.* (1997); Kulik *et al.* (1997)]. Previous studies have revealed that PI 3-kinase is involved in growth factor-dependent survival of PC12 cells [Yao and Cooper (1995)] and also mediates insulin like growth factor-dependent survival of Rat-1 and COS-7 cells [Kulik *et al.* (1997)]. Recent evidence has demonstrated that PKB is both a downstream effector target of PI 3-kinase and a key mediator of growth factor induced cell survival and protection against c-Myc induced cell death in fibroblasts [Kauffmann-Zeh *et al.* (1997)]. The exact meachanism by which CD28 prevents apoptosis however are not yet determined. One possibility is that PKB may be involved in the upstream signalling events which determine the CD28 augmented expression of the cell survival factor Bcl$_{XL}$. This may be analagous to the situation observed in IL-3 signalling whereby, in haematopoietic cells the cytokine IL-3, under conditions where it promotes cell survival, induces the phosphorylation of BAD at ^{112}Ser and ^{136}Ser [del

Peso *et al.* (1997)]. In the absence of phosphorylation of these sites, BAD is thought to induce cell death by the formation of heterodimers with BclXL, and the concomitant generation of BAX heterodimers. The IL-3 mediated phosphorylation of either the ^{112}Ser or the ^{136}Ser site may promote the survival of haematopoietic cells since phosphorylation of BAD has been correlated to its sequestration by the cytosolic protein 14-3-3 [Zha *et al.* (1996)], liberating the cell survival factor BclXL.

PKB activation is known to have at least one further major outcome which may be relevant to the functional outcome of CD28 ligation in T cells, namely p70 S6 kinase activation [Burgering and Coffer (1995)], an event which has also been demonstrated to follow CD28 ligation. However the significance of CD28 induced activation of p70 S6 kinase has not yet been fully investigated. Whilst not sufficient to drive proliferation, the induction of p70 S6 kinase activity may be necessary for the intitiation of cell cycle progression in resting T cells. Blocking of p70 S6 kinase either by injection of Ab or by the use of rapamycin has suggested a role for p70 S6 kinase in the G1-S phase transition [Lane *et al.* (1993); Reinhard *et al.* (1994)]. Considering the evidence that p70 S6 kinase is a downstream element of the PI 3-kinase signalling cascade [Burgering and Coffer (1995)], a role for CD28 induced signals in the activation of p70 S6 kinase seems appropriate, particularly given the demonstration that CD28 driven T cell proliferation requires the activation of PI 3-kinase [Ward *et al.* (1995)]. Interestingly, rapamycin which potently inhibits p70 S6 kinase activation, also inhibits anti-CD28 mAb induced IκBα down-regulation and c-rel translocation in Jurkat cells and primary T cells [Lai and Tan (1994)], indicating that regulation of transcription factors involved in CD28 dependent regulation of cytokine/chemokine genes may involve the PI 3-kinase dependent signalling cascade. There is evidence however that this may not be the case in normal T-lymphoblasts since experiments using human T-lymphoblasts indicate that CD28-induced activation of NFκB is unaffected by pretreatment with PI 3-kinase inhibitors [Edmead *et al.* (1996)]. Further downstream targets for PKB exist in glycogen synthase kinase-3 (GSK-3) [Cross *et al.* (1995)], which is serine phosphorylated and thus inactivated by PKB allowing the molecule to influence glycogen metabolism. Since PKB is activated by CD28, the idea that PKB regulates several downstream targets is consistent with the fact that many functional outcomes have been attributed to CD28, such as regulation of multiple cytokine/chemokine genes and protection against apoptosis.

7.3.2 CD28 activates p70 S6 kinase.

Activation of p70 S6 kinase is known to involve the phosphorylation of at least nine phosphorylation sites and it appears that activation may be sequential in phosphorylation of proline-directed sites in the carboxy-terminal tail, followed by phosphorylation of ^{389}Thr and ^{229}Ser to yield a fully active kinase [Pullen and Thomas (1997)]. Despite the importance of these phosphorylation sites, until very recently, the identity of the kinases responsible for the phosphorylation of these residues remained unknown. In this study the PI 3-kinase inhibitor wortmannin has been used to assess the role of PI 3-kinase signalling to couple CD28 to p70 S6 kinase. Treatment of Jurkat cells with the PKC-activating phorbol ester, phorbol-12-myristate-13-acetate (PMA) resulted in the activation of p70 S6 kinase, which was inhibited by pretreatment with rapamycin, confirming previous observations [Chung *et al.* (1994)]. Rapamycin exerts its inhibitory effect when complexed with its cytosolic target protein FKBP. The inhibitory effect of rapamycin can be overcome by expression of a mutated version of mTOR that fails to bind the FKBP-rapamycin complex, indicating that mTOR is an upstream component of the pathways controlling p70 S6 kinase [Brown *et al.* (1995)]. Stimulation of Jurkat cells with anti-CD28 mAb also resulted in the activation of p70 S6 kinase. This CD28-dependent activation of p70 S6 kinase was inhibited by pretreatment with rapamycin, but resistant to pretreatment with the immunosuppressant cyclosporin A, suggesting p70 S6 kinase activation to be independent of the function of the phosphatase calcineurin, and correlating with the original demonstration that CD28 biochemical signals required for T cell proliferation are cyclosporin A-resistant [June *et al.* (1987)]. Pretreatment of Jurkat cells with nanomolar concentrations of wortmannin, previously shown to inhibit CD28-induced accumulation of the D-3 phosphoinositides (section 3.1), resulted in inhibition of CD28 induced activation of p70 S6 kinase. The observation that rapamycin, which potently inhibited CD28-mediated p70 S6 kinase activation, had no effect on the accumulation of PtdIns(3,4,5)P_3, suggests that the effects of rapamycin were not due to upstream inhibition of PI 3-kinase, and that rapamycin must function by inhibition of target(s) downstream of, or on separate pathways from PI 3-kinase. Thus, these data imply that either i) wortmannin and rapamycin inhibit CD28-mediated activation of p70 S6 kinase at distinct points along the PI 3-kinase-dependent signalling pathway, or ii) rapamycin inhibits a further pathway required for p70 S6 kinase activity, and iii) activation of p70 S6 kinase is at least one downstream target for the PI 3-kinase signalling pathway following CD28 ligation on T cells.

Evidence for PI 3-kinase and mTOR forming components of separate cell signalling pathways is provided by the observation that removal of the NH_2-terminal region of p70

S6 kinase, results in a truncated kinase that is resistant to inhibition by rapamycin but still inhibited by wortmannin [Weng *et al.* (1995)]. However, this does not discount the possibility that wortmannin also targets the catalytic domain of mTOR itself, which displays homology to the catalytic domain of PI 3-kinase [Brown *et al.* (1994); Sabatini *et al.* (1994)]. Indeed, wortmannin and LY294002 have been demonstrated to inhibit the protein serine kinase activity of mTOR [Brunn *et al.* (1996)] at concentrations corresponding to those required for inhibition of the lipid kinase function of PI 3-kinase, although this has not been consistently observed [Brown *et al.* (1995)]. Despite the reported homology with PI 3-kinase however, a lipid kinase function for mTOR has not been reported to date [Brown *et al.* (1995)].

The approach adopted in this study, to examine the regulation of p70 S6 kinase, by immunoblot analysis for electrophoretically retarded isoforms of p70 S6 kinase was intially attractive since it offered a simple protocol to graphically illustrate the activation of p70 S6 kinase. The nature of the protocol however entails a number of inherent draw backs. Firstly, since CHO-B7.1^{+} cells would have contributed proteins that would be recognised in Western blots, cell stimulations were performed using anti-CD28 mAb, which may lead to results discrepant from those obtained by ligation of CD28 by its natural ligand. Secondly, the protocol is not conducive to obtaining quantitative data. Expression of an HA-tagged p70 S6 kinase protein, followed by stimulation of cells with the natural CD28-ligand B7.1, and *in vitro* kinase assay, may have allowed for more quantitative data to be obtained.

Subsequent investigations have shown that the recently described kinase termed PDK1, which is capable of phosphorylating PKB on ^{308}Thr [Alessi *et al.* (1997)], is also capable of phosphorylating p70 S6 kinase on ^{229}Thr [Pullen *et al* (1998)], an event which appears essential in the activation of the enzyme [Pullen and Thomas (1997)]. PDK1 appears to be constitutively active and *in vitro* experiments have shown immunoprecipitated Myc-tagged PDK1 to phosphorylate p70 S6 kinase in a manner that is insensitive to wortmannin [Pullen *et al.* (1998)], correlating with the wortmannin insensitive phosphorylation of PKB by PDK1 *in vitro* [Alessi *et al.* (1997)]. Despite the constitutively active nature of PDK1, the molecule may be dependent upon PtdIns(3,4,5)P_3 for its function, in phosphorylating PKB, since binding of the lipid to the PH domains of both PDK1 and PKB results in their translocation to the membrane [Anderson *et al.* (1998)]. Drawing an analogy with PKB, positional information may also be important in the activation of p70 S6 kinase, indeed, nuclear localisation of the related p85 S6 kinase appears to be a functional requirement for the molecule [Reinhard *et al.* (1994)]. If PDK1 function in phosphorylating p70 S6 kinase were to be similarly

dependent upon the lipid products of PI 3-kinase, PDK1 would be placed as an intervening molecule between PI 3-kinase and p70 S6 kinase, and provide at least one explanation for the wortmannin sensitivity of CD28 mediated activation of p70 S6 kinase.

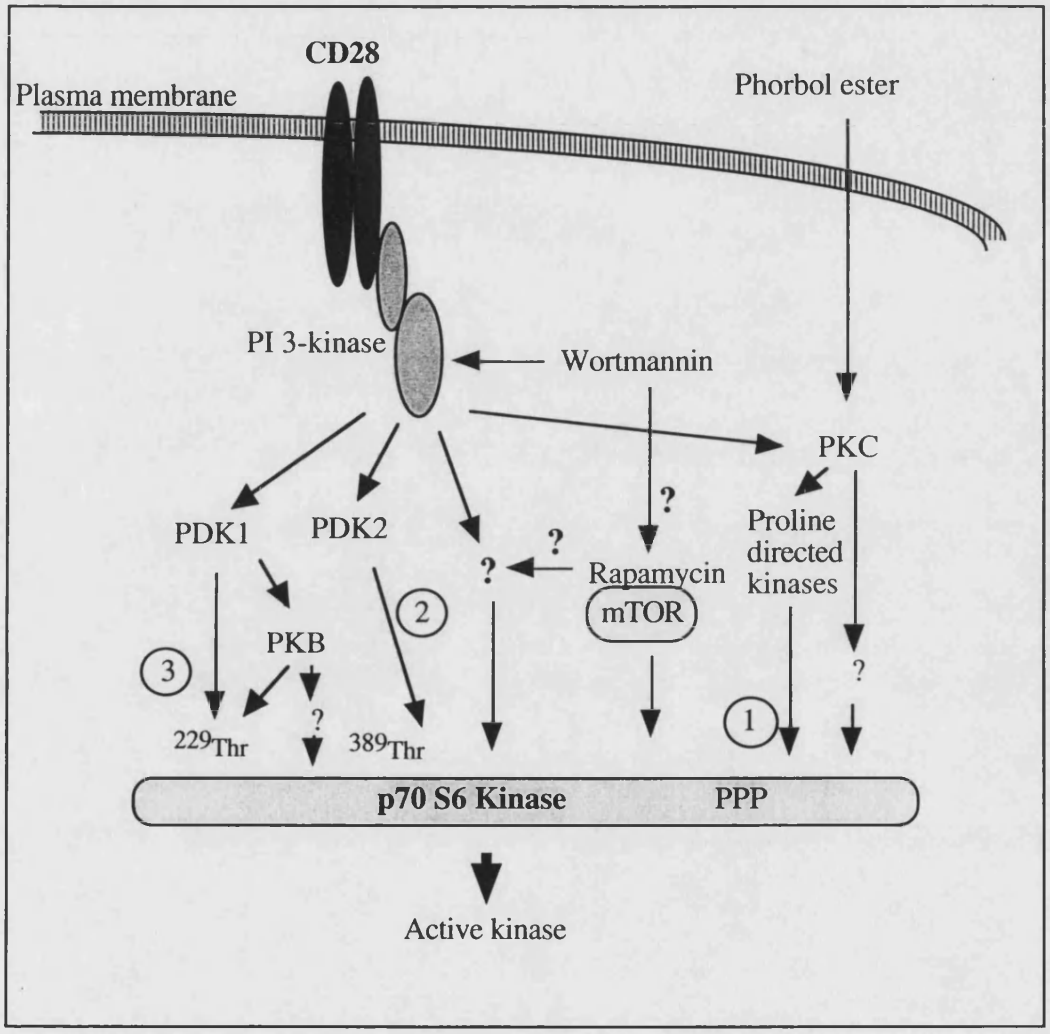


Figure 7.4 CD28-mediated activation of p70 S6 kinase in T cells. Ligation of CD28 by mAb was found to induce activation of p70 S6 kinase, which was sensitive to inhibition by wortmannin and rapamycin. Phorbol ester treatment of cells also stimulated activation of p70 S6 kinase. Activation of p70 S6 kinase *in vivo* is thought to involve sequential phosphorylation 1) initially by proline-directed kinases in an autoinhibitory region [Ferrari *et al.* (1992)], then 2) by a PtdIns(3,4,5)P_3-dependent kinase at ^{389}Thr (sequence data suggests this to be same kinase as the putative PDK2) inducing a conformational change exposing ^{229}Thr for 3) phosphorylation by PDK1 [Pullen *et al.* (1998)] to yield an active kinase. Thus the mechanism of activation of p70 S6 kinase shares many common themes with the activation of PKB [Reviewed by Downward (1998)]. The nomenclature of residues is used as for the p70 S6 kinase αII isoform, whilst identical phosphorylation sites exist in the p70 S6 kinase αI isoform.

Further analogy can be drawn between the activation of PKB and p70 S6 kinase since the ^{389}Thr phosphorylation site within p70 S6 kinase is analogous to the ^{473}Ser residue of PKB, which is phosphorylated by the putative kinase PDK2. As is the case for PKB phosphorylation of both these sites causes a synergistic activation of kinase activity. Thus PKB and p70 S6 kinase apparently share common mechanisms of activation and upstream kinases. Distinct from PKB however, p70 S6 kinase is initially required to be phosphorylated by proline directed kinases, such as MAP kinases, which phosphorylate a number of sites in the putative autoinhibitory carboxy terminus of p70 S6 kinase [Ferrari *et al.* (1992)]. The data presented in this thesis and subsequent data from the literature are summarised in Figure 7.4.

7.3.3 CD28 activates SHIP.

The lipid products of PI 3-kinase represent a minor, but very important signalling component of the cell membrane. Levels of these lipids therefore, need to be tightly regulated. The *myo*-inositol lipids are subject to degradation by lipid phosphatases which limit the availability of the putative signalling molecules. The D-5 phosphatases are the most well characterised PI lipid phosphatases and SHIP is one such inositol polyphosphate 5'-phosphatase, which exhibits a unique substrate specificity in that it hydrolyses only substrates phosphorylated at the D-3 position i.e. Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3. This study has shown using *in vitro* protein kinase assay, that SHIP is phosphorylated following CD28 ligation and, immunoblotting with anti-phosphotyrosine Ab revealed that this phosphorylation resides within tyrosine residues of SHIP. Moreover, the functional activation of SHIP has been demonstrated following ligation of CD28 by *in vitro* SHIP assays using Ins(1,3,4,5)P_4 as a substrate and monitoring the formation of Ins(1,3,4)P_3. These results are in marked contrast to those of Osborne *et al.* who showed that tyrosine phosphorylation of SHIP by lck expressed in *S. cerevisiae* resulted in a inhibition of phosphatase activity [Osbourne *et al.* (1996)]. This discrepancy may reflect the heterogeneity within the cellular models utilised. Additionally, when the authors immunoprecipitated SHIP from RBL-2H3 cells they reported a high basal state of SHIP tyrosine phosphorylation which obscured any receptor mediated increase in phosphorylation [Osborne *et al.* (1996)]. In contrast this study and other reports [Giuriato *et al.* (1997); Kimura *et al.* (1997)], have clearly demonstrated receptor mediated tyrosine phosphorylation of SHIP. Differences in the biochemistry of the cellular models employed may therefore explain the functional discrepancies observed. The results presented in this study, and those of Giurato *et al.* (1997), who demonstrated thrombin activation of SHIP in platelets, argue against the

notion that only negative signalling conditions, such as co-crosslinking of the B cell antigen receptor and FcγR, promote tyrosine phosphorylation of SHIP [Tridandapani *et al.* (1997)].

SHIP can bind directly to a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) present in the cytoplasmic domain of FcγRIIB [Ono *et al.* (1996)], and association has been reported between SHIP and immunoreceptor tyrosine-based activation motifs (ITAM) present in the CD3 complex and TCR ζ chains. Despite the tentative description of CTLA-4 as an ITIM bearing molecule, on the basis of its association with the phosphatase SHP-2 however [Vivier and Daeron (1998)], neither CTLA-4 or CD28 encode any recognised ITAM or ITIM motifs. The mechanism by which SHIP actually couples to CD28 is obscure although there are several possibilities. These include interactions with intermediate adaptor proteins via its: i) SH2 domain, ii) proline rich regions or iii) the two NPXY motifs which bind phosphotyrosine motifs. An example of possible intermediate adaptor proteins is found in Grb-2 since this molecule has been previously demostrated to associate with CD28 [Schneider *et al.* (1995a)] and SHIP is known to associate with Grb-2 via an interaction between its proline rich region and SH3 domain of Grb-2 [Kavanaugh *et al.* (1996)]. However, whilst this is an attractive hypothesis, under conditions where CD28 co-association with p85 can be detected, no association of CD28 with SHIP was detected. Moreover, the association of Grb-2 with CD28 in DC27.1 cells has not been demonstrated to date (Daniel Olive, personal communication). *In vitro* kinase assays of SHIP immunoprecipitates demonstrate that CD28 ligation induces the phosphorylation of a 145 kDa subtsrate, presumably SHIP, in murine DC27.1 cells. This phosphorylation is accompanied by further molecules of approximately 40 kDa and 100 kDa, the identity of which is unknown and these molecules may represent SHIP-associated protein tyrosine kinases and/or phosphorylated adaptor proteins that regulate activation and/or protein-protein associations of SHIP. Alternatively CD28 may affect SHIP activity via regulation of PLCγ1. Stimulation of CD28 by mAb in DC27.1 cells induces tyrosine phosphoylation of PLCγ1, which may receive further regulatory input from the lipid products of PI 3-kinase [Bae *et al.* (1998); Falasca *et al.* (1998)]. SHIP has been demonstrated to associate with the SH3 domain of PLCγ1 in RBL-2H3 cells [Osborne *et al.* (1996)] and this interaction may allow for CD28-mediated regulation of SHIP either by localisation of the phosphatase to regulatory proteins or substrate.

Activation of SHIP and subsequent hydrolysis of PtdIns(3,4,5)P_3 has been proposed as inhibitory to the biological effects of PI 3-kinase [Deuter-Reinhard *et al.* (1997)]. The cellular function of SHIP is a moot point however, for instance, activation of SHIP may

not represent a true "off signal" since SHIP activity results in an increase of level of PtdIns(3,4)P_2, for which there may be downstream targets such as PKB [Franke *et al.* (1997)] or PKC family members [Toker *et al.* (1994)], thus SHIP activity results in a "shunting" of the PI 3-kinase signal away from PtdIns(3,4,5)P_3 targets towards PtsIns(3,4)P_2 targets. Thus, with respect to its activation by CD28, SHIP may be i) a "house keeping" receptor-operated negative feedback mechanism which keeps tight control of PtdIns(3,4,5)P_3-dependent signalling mechanisms and down-regulates effects on mitogenesis, cellular transformation and immune function. In this respect SHIP would facilitate the controlled removal of PtdIns(3,4,5)P_3 and thus remove a membrane targetting signal for recruitment of PH domain containing signalling molecules, similar to that reported to occur during SHIP mediated inhibitory signals on PtdIns(3,4,5)P_3/Tec kinase dependent signalling in B cells after BCR ligation [Scharenberg *et al.* (1998)]. ii) Alternatively SHIP may divert signalling away from PtdIns(3,4,5)P_3-dependent effectors towards effectors exclusively driven by PtdIns(3,4)P_2 thus achieving functional diversity. It should be stressed that the notion that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 may exert different functional outcomes is contentious since several studies have revealed these lipids to have either similar or different effects in a range of *in vitro* assays such as binding of PH domains or PKB activation [James *et al.* (1996); Frech *et al.* (1997); Franke *et al.* (1997)]. This may be important in controlling the subtle mechanisms that regulate CD28 coupling to Vav, $p62^{dok}$ and/or PKB and other signalling molecules not yet identified.

The increasing number of molecules reported to utilise the PI 3-kinase mediated pathway potentially poses a problem for the cell in preserving specificity in signal transduction and elicitation of appropriate responses. Differential recruitment and/or activation of SHIP by molecules engaging the PI 3-kinase pathway may enable tailoring of the resultant signal, since SHIP will influence both duration and intensity of PtdIns(3,4,5)P_3 levels generated. Additionally SHIP will regulate the activity of its own potential downstream targets, thus recruitment and/or activation of SHIP could constitute an important regulatory mechanism in activation of downstream target effector molecules. Whether SHIP is simply inhibitory to the costimulatory signal, generated via CD28 ligation, or results in enhanced activation of identified or novel targets of its lipid products requires further investigation. A model of SHIP regulation of CD28-mediated activation of PI 3-kinase dependent signalling cascades is proposed in Figure 7.5.

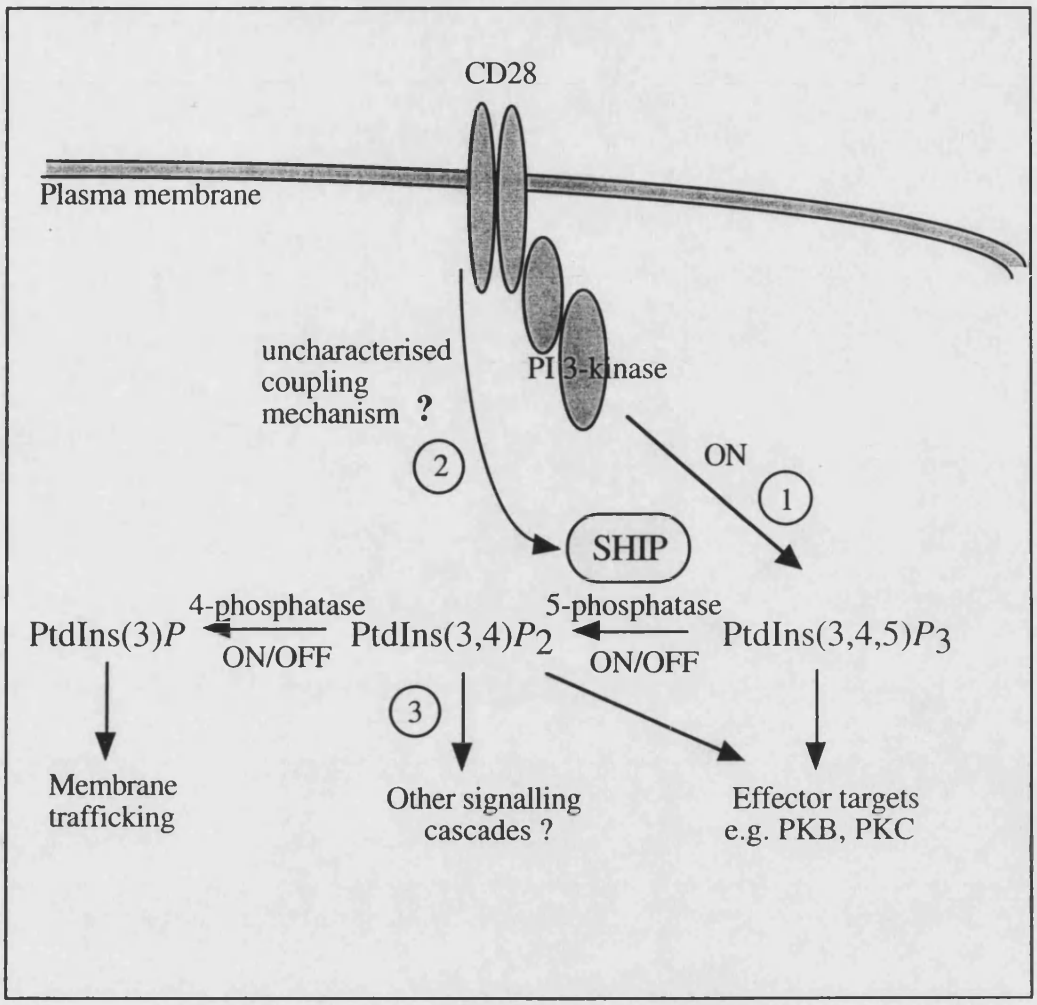


Figure 7.5 Model for regulation of CD28-activated PI 3-kinase-dependent signalling cascade by SHIP. 1) CD28 ligation induces strong activation of PI 3-kinase resulting in the accumulation of PtdIns(3,4,5)P_3 [Ward *et al.* (1993)] and consequent activation of PI 3-kinase dependent cascades. 2) CD28 ligation also induces tyrosine phosphorylation of SHIP and stimulation of its phosphatase activity. Although the mechanism of CD28 coupling to SHIP is not characterised a direct association is unlikely since no SHIP can be detected in CD28 immunoprecipitates by immunoblot analysis. 3) SHIP catalytic activity will degrade PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 although this may not represent a true "off" switch since there may be targets activated by this lipid, thus SHIP activity may "shunt" the CD28-mediated PI 3-kinase signal from PtdIns (3,4,5)P_3 targets towards PtdIns(3,4)P_2 targets.

7.4 Activation of the MAP kinases.

Of the three MAP kinase pathways described in mammalian cells, the ERK pathway is the best defined. This pathway is upregulated via Ras, which is transiently activated in response to TCR ligation. This study has used *in vitro* kinase techniques to demonstrate that JNK activation is induced by treatment of cells with CD3 mAb and CD28 mAb, or CD28 mAb in combination with PMA and ionomycin, confirming previous observations [Su *et al.* (1994)]. Furthermore, from immunoblot analysis using an antibody which recognises only the tyrosine phosphorylated form of p38, CD28 stimulation has been demonstrated to induce the activation of p38 MAP kinase. The relative contributions of ERK, JNK and p38 pathways in T cell activation cannot be determined unless specific pharmacological or molecular reagents that selectively block each of these pathways are available. In this study PD98059, a compound which specifically inhibits signalling through the ERK pathway by inhibition of MEK, and SB203580, which specifically inhibits the p38 MAP kinase, have been used to try to dissect out the effects of these pathways on T cell activation. The results are sumarised in Figure 7.6.

The MEK pathway can efficiently couple the TCR and phorbol ester-activated PKC to the ERK2 pathway [Genot *et al.* (1996)]. However the MEK inhibitor PD98059 induced only slight inhibition of CD3/CD28 driven proliferation, except at higher concentrations (e.g. <40% inhibition at 50 μM) which may compromise the specificity of the compound. The relative lack of effect of PD98059 on proliferation induced by the TCR/CD28 and CD28/PMA/ionomycin correlates well with previous observations derived from mice expressing dominant negative MEK-1 transgenes [Alberola-Ila *et al.* (1995)]. These experiments revealed that MEK activation is not required for TCR-induced proliferative responses of mature thymocytes even though $p21^{ras}$ signals are indispensable [Alberola-Ila *et al.* (1995)]. Rather, it appears that MEK activation is critical to thymocyte maturation (positive selection) and differentiation from double negative CD4/CD8 to double positive thymocytes in Recombination-activating gene $(Rag)^{-/-}$ mice [Crompton *et al.* (1996)]. The lack of effect of MEK inhibitors on T cell proliferation may be explained by a growing body of evidence indicating that ras effector pathways other than the Raf-1/MEK/ERK2 pathway exist. For instance, activation of the MEK pathway is not sufficient for ras activation of the transcription factors AP1 or NFAT in T cells [Genot *et al.* (1996)]. Similarly, in mast cells, ERK1/2 MAP kinases are not required for FcγRI regulation of NFAT at all, whilst activation of ras is essential [Turner and Cantrell (1997)]. Indeed, it appears that ras stimulates a Rac-1 mediated pathway that regulates the transcriptional function of AP1 complexes in T cells [Genot *et al.* (1998)] whilst Rac-1 is also an important ras effector involved in FcεRI regulation of the transcriptional activity

of Elk-1 and NFAT in mast cells [Turner and Cantrell (1997)].

Since phorbol ester-activated PKC can also activate $p21^{ras}$ [Downward *et al.* (1990)] similar arguments relating to the ras effectors being required for T cell proliferation can be used to explain the the lack of effect of PD98059 on PMA/CD28 and PMA ionomycin stimulated proliferation. The experiments presented in this study revealed only a slight inhibitory effect of PD98059 at higher concentrations which may indicate either i) non-selective effects of PD98059 at higher concentrations; or ii) a partial involvement of MEK in the proliferative responses of human T cells to CD28/CD3 Ab, since anti-CD28 Ab may provoke an accentuated increase in MAP kinase activation, the proliferative reponse of T cells in response to Ab stimulation may be unusually dependent upon MEK.

This study shows that the previously termed "stress activated kinase" p38 is also activated in T cells by CD28 and of integral importance to CD3/CD28 mitogenic signalling, since p38 inhibition with SB203580 correlated with inhbition of CD3/CD28 driven proliferation, although the effect of CD3 ligation on p38 activation however, was not determined. Additionally, evidence is presented that p38 is involved in transducing the mitogenic responses to IL-2. Care must be taken however, when interpreting the results from the IL-2 bioassay, which correlated inhibition of p38 to inhibition of IL-2 production in response to CD3/CD28 stimulation, since the inhibitor may be carried over in the cell supernatant which was harvested for IL-2 assay. The possibility that SB203580 inhibits another protein kinase is unlikely, as it has no activity when tested on a large number of other kinases at high concentration [Cuenda *et al.* (1995)]. However, the possible existence of non-kinase targets cannot be absolutely discounted. These results imply that p38 MAP kinase has a novel role in mediating the proliferative response of T cells to cytokine stimulation.

To determine if the inhibitory effects of SB203580 on CD28 driven proliferation could be accounted for by non-specific inhibition of PI 3-kinase, the effect SB203580 was assessed on CD28 mediated accumulation of PtdIns(3,4,5)P_3. Incubation of cells with SB203580 however was found to have no effect on CD28-mediated PI 3-kinase activation indicating that PI 3-kinase activation lies either upstream of p38 activation or on a dichotymous pathway of CD28 signalling. The latter would correlate well with subsequent data, which suggests that one possible route by which CD28 may activate the p38 pathway is via the activation of p21-activated kinase (PAK) [Kaga *et al.* (1998)]. The activation of PAK has been proposed as a point of integration of the CD3 and CD28 signals, whilst under these conditions the signal provided by CD28 can be mimicked by treatment of cells with ceramide analogues [Kaga *et al.* (1998)]. These data imply that

CD28 may supply a PAK activating signal that is independent of PI 3-kinase. However, PI 3-kinase has previously been implicated in the insulin-mediated activation of PAK in muscle cells [Tsakiridis *et al.* (1996)], hence PAKs may be regulated by both ceramide and PI 3-kinase mediated signals. PI 3-kinase appears play some role in the activation of JNK, as evidenced by the wortmannin-mediated partial inhibition of anti-CD3 plus anti-CD28 Ab stimulated of JNK in Jurkat cells demonstrated in this thesis, which correlates with the reported requirement for PI 3-kinase activity in growth factor mediated JNK stimulation [Logan *et al.* (1997); Lopez-Ilasaca *et al.* (1997)], and JNK activation in COS cells [Klippel *et al.* (1996)]. This may be a cell-specific phenomenon however, since previous results have described that PI 3-kinase cannot stimulate JNK in NIH3T3 cells [Reif *et al.* (1996)].

CD28 may couple Rac/Rho effector PAK via regulation of $p62^{dok}$ and $p97^{vav}$ which are both tyrosine phosphorylated on CD28 ligation [Klasen *et al.* (1998)]. $p62^{dok}$ forms a complex with p120 RasGAP and p190 RhoGAP and it is postulated that tyrosine phosphorylation of $p62^{dok}$ will modulate the cellular distribution of RasGAPs and RhoGAPs and sequester these molecules away from active ras and rac molecules, thereby prolonging the activation of Rac or Rho dependent pathways. Additionally, both dok and vav encode PH domains which may allow CD28 activated PI 3-kinase to induce either allosteric modification or membrane translocation of these molecules via binding of D-3 lipids to their PH domains.

An emerging theme in the literature suggests that p38 and JNK/SAPK may play decisive roles in the control of cell death. This is supported by the observation that transfection of a constitutively active mutant of MKK3/6, the physiological activator of p38, along with p38 is sufficient to induce apoptosis in PC12 cells [Xia *et al.* (1995)]. Additionally, transfection of dominant negative mutants of MKK3/6 in PC12 cells or of p38 in NIH-3T3 fibroblasts dramatically inhibits apoptosis by nerve growth factor withdrawal [Xia *et al.* (1995)] and U.V. irradiation [Berra *et al.* (1997)]. Interestingly, deprivation of neutrophic factors in PC-12 or UV-irradiation of NIH-3T3 cells not only activates the stress kinase cascades, but also leads to a dramatic inhibition of the ERK pathway [Xia *et al.* (1995); Berra *et al.* (1997)]. This is particularly relevant since ERK has been shown to be required for survival signalling in response to fibroblasts growth factor [Gardner and Johnson (1996)] and insulin like growth factor 1 [Parrizas *et al.* (1997)]. Thus it seems that the ability of a cell to die or survive may be dictated by a critical balance between the ERK and the JNK/SAPK and p38 pathways [Canman and Castan (1996)]. Given the role of CD28 in determining cell survival, it seems entirely appropriate that it can regulate the pathways known to play important roles in cell survival and/or cell death.

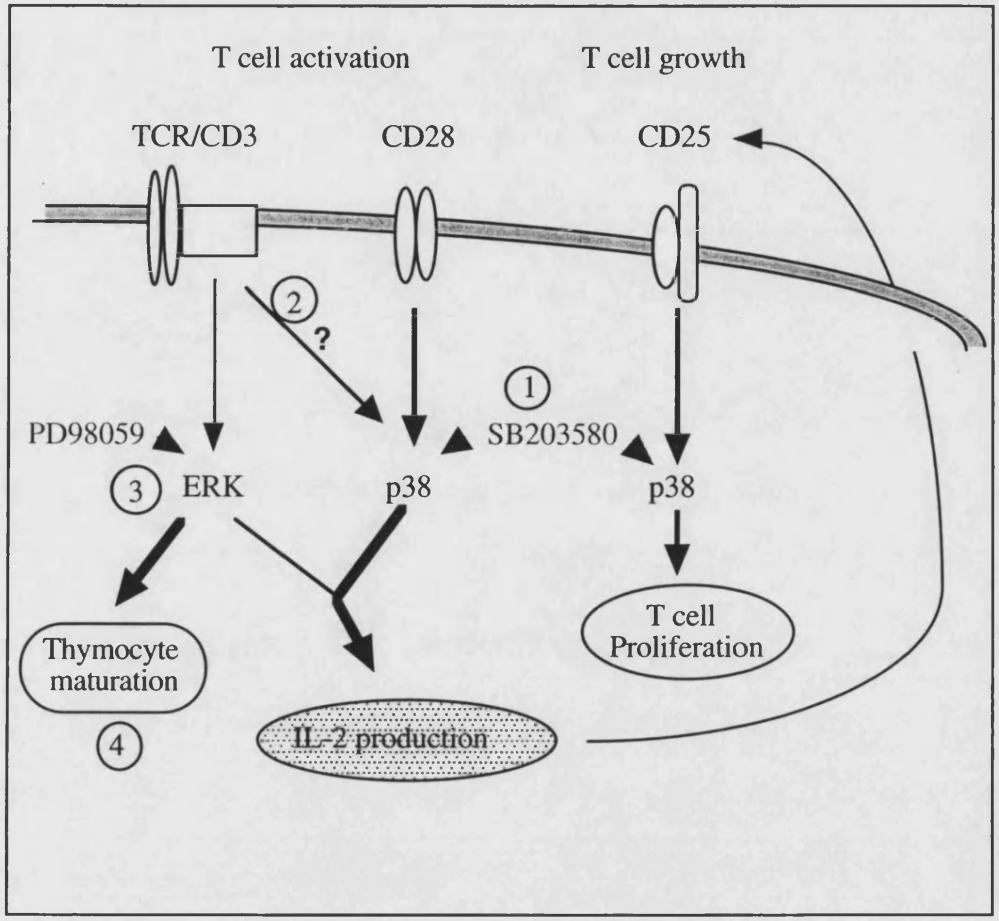


Figure 7.6 Diagrammatic summary of results presented in section 5.4. 1) Inhibition of p38 MAP kinase using SB203580 correlated with inhibition of [^{3}H]-thymidine incorporation in response to TCR/CD3 plus anti-CD28 mAb or anti-CD28 mAb plus PMA or IL-2. 2) The effect of TCR/CD3 ligation on p38 stimulation however, was not determined. These data indicate a novel role for the previously termed "stress activated" kinase in T cell activation and proliferation. 3) In contrast the MEK inhibitor PD98059 was found to inhibit T cell [^{3}H]-thymidine incorporation driven by TCR/CD3 plus CD28 only partially at higher concentrations of the drug, which may be explained by inhibition of non-specific targets. [^{3}H]-thymidine incorporation driven by IL-2 or anti-CD28 mAb plus PMA was not inhibited by PD98059. 4) The apparent redundancy of ERK in proliferative responses may suggest further cellular functions for the pathway, for example studies suggest an important role in thymocyte maturation [Alberola-Ila *et al.* (1995)]. Size of arrow indicates inferred importance of pathway to functional outcome.

7.5 CD95 activation of costimulatory targets.

Given the crucial role of CD95 function in the immune system, the events linking CD95 ligation to apoptosis have been the subject of intense investigation. Reports have demonstrated that CD95 employs a number of signal transduction pathways such as activation of the caspases [Enari *et al.* (1995)], which may be peculiar to apoptotic pathways. Other CD95 targets however are also activated by mitogenic signals. For example, it has also been reported that ceramide levels increase after CD95 ligation [Cifone *et al.* (1994)], and CD28 ligation [Boucher *et al.* (1995)], and Fas has been demonstrated to couple to fyn, a PTK pivotal to T cell activation. Given the contrasting agendas of the apoptotic and stimulatory pathways, it might be assumed that the effect of CD95 ligation on costimulatory targets would either be negligible or inhibitory. However, considering that under certain circumstances Fas may be implicated as providing a costimulatory signal (as detailed in section 1.8) the effect of Fas ligation was investigated on signalling targets known to be stimulated by mitogenic stimuli.

7.5.1 CD95 activates PI 3-kinase and downstream effectors.

PI 3-kinase has been implicated in a wide range of cellular functional responses, see Table 1.3. The data presented in this study imply that PI 3-kinase is also activated by cell death-inducing receptors since ligation of CD95 is shown to induce the accumulation of the lipid products of PI 3-kinase (section 6.2). The activation of PI 3-kinase is not necessary for efficient death signalling however, since treatment of Jurkat cells with the PI 3-kinase inhibitors wortmannin and LY294002 potentiated, rather than inhibited, CD95-mediated cell death. This also correlated with increased CD95-mediated cell death seen in JCaM1 cells which display severely impaired activation of PI 3-kinase. These observations are summarised in Figure 7.7.

The magnitude of CD95-induced PI 3-kinase activation is markedly lower than that observed on CD28 ligation. However, the D-3 phosphoinositides, particularly PtdIns(3,4,5)P_3, are generally restricted to the membrane compartments in which they are made, leading to a high degree of compartmentalisation, allowing for high concentrations of the lipids to be encountered locally which may enable activation of downstream effectors of PI 3-kinase. Given this, investigations were carried out to see if CD95-mediated activation of PKB was sufficient to activate downstream effectors of PI 3-kinase.

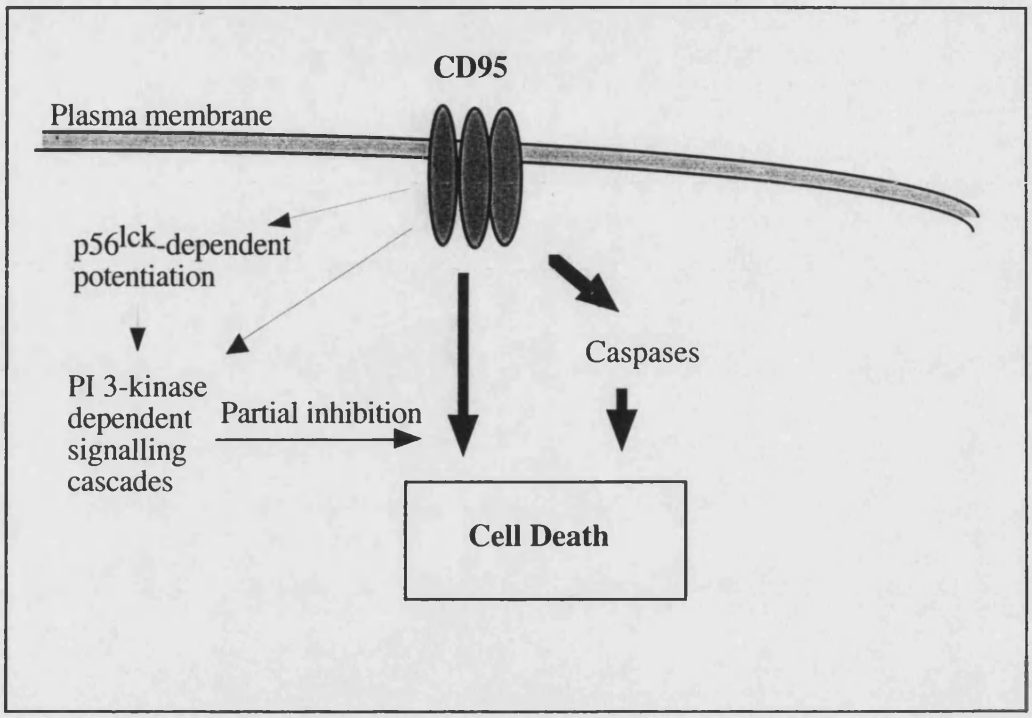


Figure 7.7 PI 3-kinase activation antagonises cell death. PI 3-kinase inhibitors LY294002 and wortmannin potentiated, rather than inhibited, CD95-mediated cell death, thus PI 3-kinase may antagonise cell death. This correlates with increased CD95-mediated cell death seen in JCaM1 cells, which fail to express $p56^{lck}$ or induce substantial CD95-activated PI 3-kinase. Size of arow indicates inferred importance of pathways to CD95 signal trnsduction

The elevation of PtdIns(3,4,5)P_3 in response to either anti-Fas Ab or Fas Ligand was found to correlate with an increased activation of the PI 3-kinase effector PKB as assessed by *in vitro* kinase assays. CD95 activation of PI 3-kinase offers the best established route for CD95 regulation of PKB activity that is, via direct binding of the lipid products of PI 3-kinase to PKB inducing a conformational change and co-localisation of PKB with PKB activating upstream kinases, such as PDK1 [Anderson *et al.* (1998)]. Attempts to demonstrate this routing by the use of wortmannin however, yielded inconclusive data since both stimulation and inhibition of CD95-mediated PKB activation was observed. These results lead to the consideration that CD95 may mediate PKB activity by signalling pathways independent of PI 3-kinase. PKB regulatory pathways independent of PI 3-kinase clearly exist, since activation of PKB is reported to be achieved by cellular stresses such as heat shock and hyperosmolarity, and this

activation appears insensitive to inhibition by wortmannin [Konishi *et al.* (1996)]. Additionally, cAMP has been shown to stimulate PKB in a wortmannin-independent manner [Sable *et al.* (1997)]. The p38 MAP kinase pathway which can be activated in response to cellular stresses is activated by CD95 [this study; Juo *et al.* (1997)], and the downstrean target of p38 namely, mitogen-activated protein kinase 2 (MAPKAP 2) can phosphorylate PKB on ^{473}Ser, at least *in vitro* [Alessi *et al.* (1996)], and might therefore contribute to the activation of PKB by cellular stress. It should be noted however, that anisomycin, a good activator of MAPKAP kinase 2, does not activate PKB [Alessi *et al.* (1996)], thus CD95-mediated activation of PKB may require input further to p38 activation. Considering the potential role of p38 in CD95 mediated activation of PKB, cells were preincubated with a specific inhibitor of p38, namely SB203580. These experiments demonstrated at least a partial inhibition of CD95 mediated PKB activation, in a dose dependent manner, implying that p38 activation by CD95 may be a contributory factor in regulation of PKB. The incomplete nature of inhibition of CD95-mediated p38 activation may be explained by the existence of SB203580 resistant isoforms. Indeed, to date, four members of the p38 group of enzymes have been described in p38, p38β, p38γ and p38δ. The most recently described group member p38δ, has been demonstrated to be expressed in Jurkat cells, and characterised as completely resistant to inhibition by pyridinyl imidazole inhibitors [Jiang *et al.* (1997)].

However, there is a strong line of evidence to suggest that CD95 does not activate PKB via p38. For instance, maximal activation of p38 was achieved at two hours following ligation of CD95, whilst the time course of CD95 mediated p38 activation was much quicker with maximal activation after five minutes. However, activation of PKB may be achieved by p38 activity occuring below the sensitivity of the assay used in this study. The CD95 activation of p38 was found to be insensitive to the presence of Z-VAD-FMK, whereas others have reported this activation to be completely inhibited by caspase inhibitors [Juo *et al.* (1997)]. This inconsistency may be explained in a number of ways: Firstly, the inhibitor may have been degraded during storage. This seems unlikely since the compound is essentially a tripeptide which has previously been stable whilst stored at -20°C and retains its ability to inhibit CD95-induced cell death. Secondly, the discrepancy may be explained by subtle differences in the biochemistry of the cell models used since Juo *et al.* used a Jurkat subclone A3, which has been selected for its susceptibility to CD95-mediated apoptosis [Juo *et al.* (1997)]. Certainly caspase independent mechanisms exists for activation of p38 since activation by sorbitol and etoposide is resistant to YVAD-FMK and Z-VAD-FMK [Juo *et al.* (1997)], and these mechanisms may be differentially regulated in Jurkat A3 cells. Thirdly, further CD95-activated control mechanisms may exist which also regulate PKB. The integrity of the PH

domain has been found to be essential for the activation of PKB in intact cells in response to activated PI 3-kinase [Franke *et al.* (1997)], and heat shock [Konishi *et al.* (1996)]. This points to an essential role for the PH domain in the activation of PKB by PI 3-kinase dependent and independent mechanisms. Structural studies have shown that the PH domain is distantly related to the immunosuppressant FK506 binding protein (FKBP), and it is possible that FKBP associates with a heat shock protein, hsp90 [Callebaut *et al.* (1992)]. It is therefore a possibility, that some heat shock proteins may bind to the PH domain of PKB, allowing stress activated pathways to regulate PKB activity. Many further proteins may associate with the PH domain of PKB, in particular, two isoforms of PKC have been demonstrated to associate with the PKB PH domain in PKCδ [Konishi *et al.* (1996)], and PKCζ [Konishi *et al.* (1994)]. Both of these PKC isoforms are activated by the lipid products of PI 3-kinase [Nakanishi *et al.* (1993); Toker *et al.* (1994)]. Intriguingly however, in the context of CD95 signalling, PKCζ has been demonstrated as activated by ceramide signals [Lozano *et al.* (1994)], and PKCδ has been reported as activated by proteolytic cleavage resultant from caspase activity [Emoto *et al.* (1995)]. These relationships are summarised in the Figure 7.8. Attractively, each branch of this signalling scheme offers a point for pharmacalogical intervention: (i) since PI 3-kinase dependent signals can be inhibited by the use of wortmannin [Thelen *et al.* (1994)] or LY294002; (ii) caspase dependent signals are subject to inhibition by cell soluble fluoromethylketone peptides Z-VAD and YVAD [Sarin *et al.* (1996)], or expression of the viral protein CrmA; (iii) p38 dependent signals would be subject to inhibition by SB203580, and (iv) ceramide dependent signals can be inhibited by the use of chloroquine (although this is a relatively non-specific inhibitor). It may be possible that CD95 mediated activation of PKB requires the presence of one or more of these signals, explaining the inconclusive results obtained in section 6.3.2. Clearly, the signalling mechanisms by which CD95 activates PKB require further examination. The rationale for CD95 activation of PKB seems hard to justify given the two such incongruous functions ascribed to the respective molecules [Itoh *et al.* (1991); Haussler *et al.* (1998)]. Transient activation of PKB could serve to "prime" downstream targets of CD95, certainly PKB can interact with members of the Bcl family of proteins, and further undefined PKB targets may exist. Alternatively, it may be that CD95 mediated PKB activation is a transient phenomenon, redundant in death signalling pathways. Certainly, PI 3-kinase function appears unnecessary, or inhibitory, to efficient death signalling in Jurkat cells. A function for PKB activation in CD95 signalling therefore, remains to be defined.

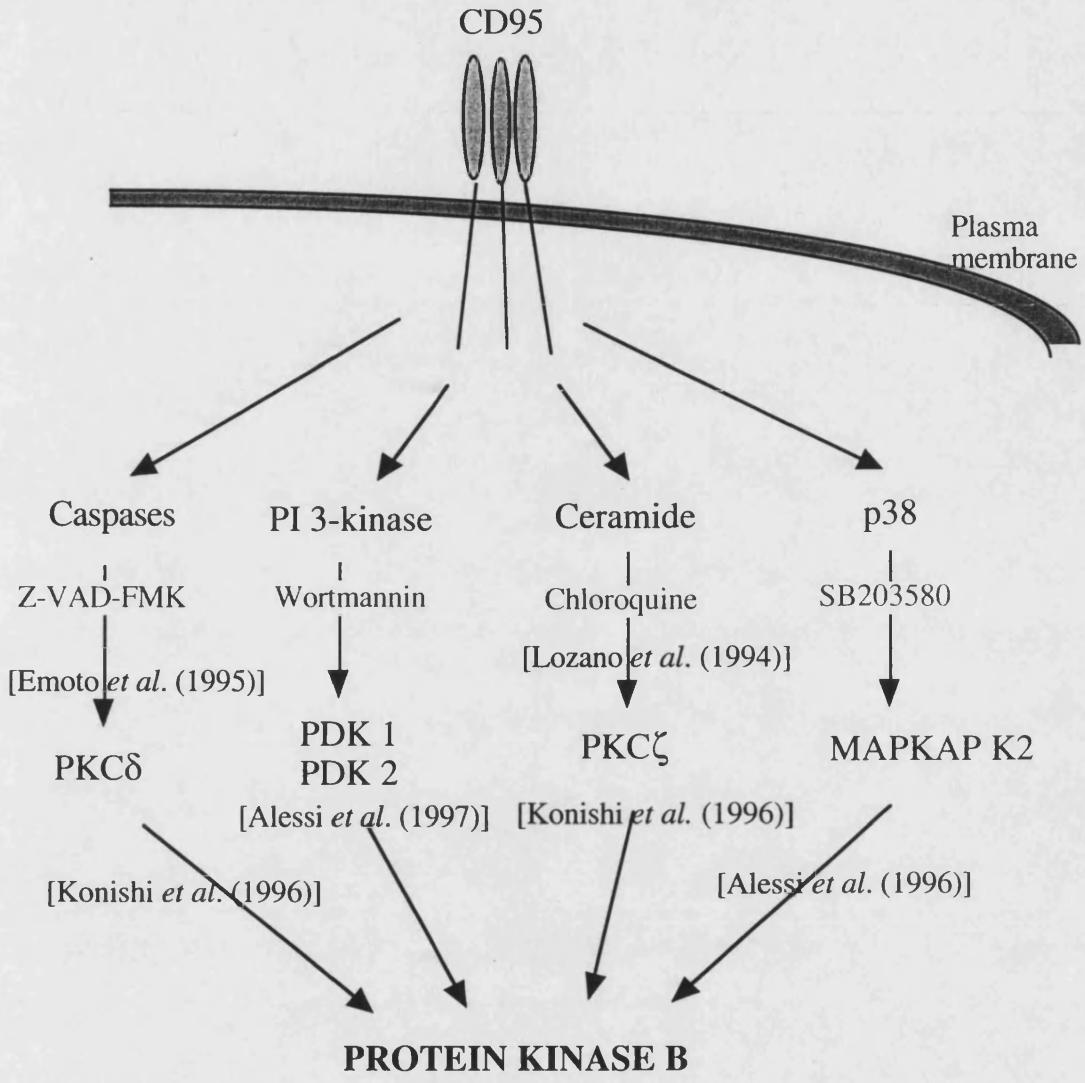


Figure 7.8 Potential routes for CD95 regulation of PKB activity. CD95 activation of PI 3-kinase provides the most obvious route for activation of PKB, however, experiments using wortmannin proved inconclusive since both potentiation and inhibition of PKB activity was observed. Thus further pathways, represented above, may couple CD95 to PKB. PKCδ and ζ isoforms have been demonstrated to interact with the PKB PH domain and may regulate PKB activity. Compounds marked in red represent potential points for pharmacological dissection of these pathways. Activation of ceramide [Cifone *et al.* (1994)] and caspases [Enari *et al.* (1995)] are recognised features of CD95 signalling. Activation of PI 3-Kinase (section 6.2) and p38 (section 6.4) following CD95 ligation has been demonstrated in this study.

7.6 SUMMARY

From the work presented in this study a number of conclusions can be drawn.

1) Lck function is not necessary for PI 3-kinase recruitment to CD28, however, it is required for efficient CD28-mediated activation of PI 3-kinase, as evidenced by the severely impaired accumulation of PtdIns(3,4,5)P_3 in JCaM1 cells.

2) PMA-activated PKC inhibits PI 3-kinase association with, and activation by CD28 following ligation by the natural ligand B7.1. Despite the presence of several phosphorylation sites within the cytoplasmic tail of CD28, the inhibitory effect of PMA on CD28/PI 3-kinase association is not mediated via modulation of the CD28 phosphorylation profile since treatment of cells with PMA, PKC inhibitors or transient expression of constitutively active PKC isoforms does not affect CD28 phosphorylation.

3) Ligation of CD28, by the natural ligand B7.1, is followed by heavy phosphorylation within serine/threonine residues of the CD28 cytoplasmic tail, which occurs independently of PI 3-kinase and PKC and is not detectably affected by a panel of inhibitors of known serine/threonine kinases.

4) Truncation and point mutation of the CD28 cytoplasmic tail reveals ligation-stimulated phosphorylation of CD28 to be dependent upon the integrity of ^{173}Y.

5) CD28-mediated activation of PI 3-kinase results in the activation of the downstream effectors PI 3-kinase and p70 S6 kinase.

6) CD28 ligation induces tyrosine phosphorylation, and stimulates the catalytic activity of SHIP. Since no direct association could be detected with SHIP, CD28 affect SHIP activity via an undefined regulatory mechanism.

7) CD28 induces activation of p38 MAP kinase, whilst pharmacological inhibition of p38 correlates with inhibition of IL-2 driven or CD28-dependent T cell proliferation.

8) CD95 ligation induces apototic death in Jurkat cells which is potentiated by inhibition of PI 3-kinase.

9) CD95 induces activation of p38 MAP kinase after one hour, and transient activation of PKB by an uncharacterised regulatory mechanism.

7.7 FUTURE DIRECTIONS

Recruitment of PI 3-kinase to CD28.

The signal transduction pathway mediated by PI 3-kinase is suggested as pivotal to T cell activation since nanomloar concentrations of wortmannin inhibit CD28 mediated costimulation of IL-2 production in resting and activated human T cells [for review see Ward *et al.* (1996)]. The present study demonstrates that CD28 recruits p85α and activates Class IA PI 3-kinases in a manner at least partially dependent on the src family tyrosine kinase lck. To date however, three distinct isoforms of p110 have been identified in p110α, p110β and p110δ, and these may not be functionally equivalent for example p110δ, unlike p110α or β, does not phosphorylate the tightly associated p85 subunit, but instead harbours an autophosphorylatory capacity [Vanhaesebroeck *et al.* (1997)]. The use of antibodies raised against specific isoforms of the catalytic subunit of PI 3-kinase would enable the analysis of the contribution of these distinct isoforms in CD28 signalling, for example the use of Western blotting and *in vitro* lipid kinase techniques could determine if these molecules all associate with CD28 and are activated by CD28, in a ligation dependent manner.

CD28 phosphorylation studies.

Despite the attention of several laboratories, the identity of the tyrosine kinase which phosphorylates CD28 at the ^{173}YMNM motif remains to be resolved. Whilst *in vitro* studies suggest Lck as strongest candidate [Raab *et al.* (1995)], the absence of Lck does not affect PI 3-kinase recruitment to CD28, thus an unidentified kinase may mediate this phosphorylation. A recent report has demonstrated that a member of the Btk/Tec family of protein tyrosine kinases namely, rlk (resting lymphocyte kinase) also termed Txk, is capable of specifically phosphorylating CTLA-4 at its (p)YVKM motif [Schneider *et al.* (1998)] which can bind the SH2 domains of PI 3-kinase [Schneider *et al.* (1995)]. Rlk may also be capable of phosphorylating CD28 in an over-expression system [Schneider *et al.* (1998)]. It would be interesting to use Western blotting and *in vitro* kinase techniques to further define the relationship between CD28 and rlk by answering such questions as do the molecules associate in intact cells, and does rlk phosphorylate CD28 *in vitro*?

The ligation dependent serine/threonine phosphorylation of CD28 has received less attention than the tyrosine phosphorylation. In the absence of any effective, specific inhibitory strategy its effect on CD28 signal transduction cannot be determined however, both enhancement by stabilising protein-protein interactions, or inhibition by disruption of CD28 coupled signalling pathways in a manner similar to that proposed to operate in insulin signalling [Shepherd *et al.* (1998)] may be envisaged. Given the pivotal role of

CD28 signal transduction to T cell activation and thus immune function, and considering that this phosphorylation may be resultant from the activity of a specific kinase since it was resistant to a panel of inhibitors of known serine/threonine kinases, the nature and identity of the CD28 directed serine/threonine kinase becomes an important issue. The ^{177}T residue is suggested as an important substrate by the present study since truncation mutation of this residue abrogates CD28 serine/threonine phosphoryation. Given the non-specific nature of deletion mutation, the significance of ^{177}T should be further investigated by point mutation of CD28 and expression in DC27.1 cells to determine more specifically if ^{177}T is required for the serine/threonine phosphorylation of CD28.

CD28 activates SHIP.

The present study demonstrates the inositol phosphatase SHIP to become tyrosine phosphorylated and activated in response to CD28 ligation in murine DC27.1 cells. Whilst in B cells SHIP is known to associate directly with CD32 via an ITIM within its cytoplasmic domain [D'ambrosia *et al.* (1995)], the cytoplasmic domain of CD28 encodes no recognised ITIM, and no direct association between CD28 and PI 3-kinase could be detected. Thus the question arises as to how CD28 couples to SHIP. *In vitro* kinase assays of SHIP immunoprecipitates reported in this study, reveal SHIP to co-immunoprecipitate with four unidentified phosphoproteins which could represent intervening kinases between CD28 and SHIP. In particular, two phosphoprotein co-immunoprecipitating with SHIP, of approximately 60 kDa, could represent the protein tyrosine kinases p59^{fyn} and p56^{lck}. Western blotting of SHIP immunoprecipitates may reveal if these molecules associate with SHIP.

Since SHIP substrates include PtdIns(3,4,5)P_3 [Damen *et al.* (1996)], SHIP activity may be presumed to inhibit cellular levels of PtdIns(3,4,5)P_3, whilst increasing levels of PtdIns(3,4)P_2. Whilst no SHIP was found to be expressed in Jurkat cells, these cells have provided a useful model for the examination of ligation induced fluctuations in levels of phosphoinositol lipids within the cell, by direct chloroform extraction of these lipids followed by deacylation and anion exchange HPLC analysis. Jurkat cells could therefore provide an interesting background to study the effects upon phosphatidylinositol lipid levels of transfection of active and inactive forms of SHIP into these cells.

The SHIP substrate PtdIns(3,4,5)P_3 is essentially membrane bound by virtue of the fatty acyl side chains of phosphoinositides. This implies that in order to hydrolyse its substrate, SHIP must translocate to the membrane on activation. This ligand induced redistribution of SHIP could be determined by intracellular staining for SHIP and subsequent analysis by microsopy. Since SHIP is known to encode a PH domain

[Damen *et al.* (1996)] it would be valid to postulate that a potential mechanism for cellular redistribution of SHIP would be provided by the binding of phosphoinositide products of PI 3-kinase enabling interaction with the plasma membrane, similar to the suggested mechanism of regulation of PDK1 activity [Anderson *et al.* (1998)]. This hypothesis could be examined either by the use of mutant CD28 expressing DC27.1 cells which do not activate PI 3-kinase (i.e.DYF173 cells), or the use of pharmacological inhibitors of PI 3-kinase.

CD95 mediated activation of PKB.

The CD95 mediated activation of PKB is an interesting observation considering the incompatibility of the reported functions of the respective molecules [Itoh *et al.* (1991); Hausler *et al.* (1998)]. It may be that CD95 mediated activation of PKB is a transient phenomenon, thus a more comprehensive characterisation would be appropriate encompassing a wider timescale than that of the present study. The inconclusive data generated from the use of pharmacogical inhibitors of PI 3-kinase suggest that further pathways may be involved in the CD95 mediated activation of PKB, as discussed in section 7.5.1. The postulated involvement of caspases, p38 MAP kinase or ceramide could be examined by the use of readily available pharmacological inhibitors of these molecules in the *in vitro* kinase assays previously utilised in this study.

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